

Christer Nordström

Localization of Small-Intestinal Enzymes –
Especially Enterokinase

Lund 1973

LOCALIZATION OF SMALL- INTESTINAL ENZYMES -
ESPECIALLY ENTEROKINASE

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Disputationen kommer att ske på engelska.



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ESPECIALLY ENTEROKINASE

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This thesis is based on the following communications, referred to in the text by their Roman numerals:

- I. Nordström, C., Dahlqvist, A. & Josefsson, L.: Quantitative determination of enzymes in different parts of the villi and crypts of rat small intestine. Comparison of alkaline phosphatase, disaccharidases and dipeptidases. *J. Histochem. Cytochem.* 15:713-721 (1967).
- II. Nordström, C., Koldovský, O. & Dahlqvist, A.: Localization of β -galactosidases and acid phosphatase in the small-intestinal wall. Comparison of adult and suckling rat. *J. Histochem. Cytochem.* 17:341-347 (1969).
- III. Nordström, C. & Dahlqvist, A.: Quantitative distribution of some enzymes along the villi and crypts of human small intestine. *Scand.J. Gastroent.* In press.
- IV. Nordström, C. & Dahlqvist, A.: Rat enterokinase: The effect of ions and the localization in the intestine. *Biochim. Biophys. Acta* 242:209-225 (1971).
- V. Nordström, C. & Dahlqvist, A.: Localization of human enterokinase. *Lancet*, ii:933-934 (1972).
- VI. Nordström, C.: Enzymic release of enteropeptidase from isolated rat duodenal brush borders. *Biochim. Biophys. Acta* 268:711-718 (1972).
- VII. Nordström, C.: Release of enteropeptidase and other brush-border enzymes from the small-intestinal wall in the rat. *Biochim. Biophys. Acta* 289:367-377 (1972).

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INTRODUCTION

The small-intestinal mucosa consists of tubular crypts (crypts of Lieberkühn) in continuity with villi which project into the lumen. A continuous sheet of a single layer of columnar epithelium covers both the villi and crypts. The major known functions of the crypts are cellular proliferation and glandular secretion. The cells are generated in the crypts, from where they migrate out and up along the villus (Leblond & Stevens, 1948). At the end of their life, they are desquamated from extrusion zones at the tips of the villi into the gut lumen. The turnover time of the small-intestinal epithelial cells is only a few days; thus these cells probably have the fastest turnover rate in the body. During the migration, they undergo marked morphological and functional differentiation into mature digestive and absorptive cells. One important and highly characteristic feature in this process is the gradual development of the brush border, the cell surface facing the lumen of the gut. This part of the cell has been intensely studied over the past ten years and has proved to be a complex membranous structure with a high degree of organization for digestive-absorptive functions (Crane, 1969). The maturation of the crypt cells also results in their losing all morphological evidence of secretory activity (Trier, 1968). In addition to the principal and predominant cells in the epithelium, there are also other types of cells in small number such as the goblet cell, the enterochromaffin cell and the Paneth cell. The goblet cells, which secrete mucus, are present both in the villi and crypts, whereas the Paneth cells are found only in the bottom of the crypts. Their function is not yet finally established. Brunner glands are present in the submucosa of the upper duodenum. They open into the bottom of the crypts and are assumed to secrete a protective alkaline mucus.

As the first part of the now presented study, a method was developed for the isolation of different parts of the villi and crypts which permitted quantitative analyses of the enzyme activities of narrow cell layers from the tip of the villi to the bottom of the crypts. Various intestinal enzymes, especially the digestive, were studied in this way in rat and human intestine. The obtained distribution curves gave information concerning which stage of differentiation the enzyme activities appear and disappear during the short lifetime of the epithelial cells. Such knowledge gives a new aspect in our concept of the functional organization of the mucosa and provides facts of particular relevance to understanding of intestinal digestion and absorption.

Many of the studied enzymes were previously well known regarding specificity and other enzymological properties, localization within the cell, and physiological role. One enzyme, however, the enterokinase (enteropeptidase, E C 3.4.4.8), had been very little investigated (at least in recent

time and with modern techniques) and the localization of this enzyme was unknown. Therefore enterokinase was subjected to a more extensive study, and the second part of this thesis deals with the localization and properties of enterokinase. The mechanisms of the release of enterokinase from the intestinal wall and the site of its physiological action were given especial attention. It was also necessary to work out certain modifications in the assay method, because determination of enterokinase activity involves different problems with each animal species and each kind of tissue preparation used.

Enterokinase is an important key enzyme in the activation of the proteolytic enzymes of the pancreas, which are secreted as inactive proenzymes (zymogens), i.e. trypsinogen, chymotrypsinogen, proelastase and procarboxypeptidase. These are converted into active enzymes when the pancreatic juice reaches the duodenum. The activation process involves first cleavage of a specific peptide bond in the trypsinogen by enterokinase, leading to removal of a peptide fragment and structural rearrangements in the molecule. When active trypsin has been formed in sufficient amount as the result of these events, this enzyme in a corresponding way activates the other proenzymes. Trypsin also has the ability to activate its own zymogen (autocatalysis), but this mechanism seems to be of minor physiological importance (Hadorn et al., 1972). Thus enterokinase is a vital enzyme for the whole intestinal protein digestion, and its absence implies severe disturbance in the utilization of dietary protein (Hadorn et al., 1969).

The absence of active proteases in freshly secreted pancreatic juice was demonstrated already a century ago (Kühne, 1867; Heidenhain, 1875). Later Schepowalnikov (1899), in Pavlov's laboratory, discovered that the pancreatic juice was activated by an intestinal factor which he named enterokinase. He showed that dog pancreatic juice, after the addition of 1 - 10 % duodenal juice, hydrolyzes fibrin 3 - 4 times more rapidly than pure pancreatic juice. Pavlov (1910) talked about enterokinase as "a ferment of ferments" to stress its superior catalytic function.

For a long time after the discovery of enterokinase, there was a considerable disagreement among the scientists in the field as to its true nature. Schepowalnikov (1899), Bayliss and Starling (1904), Vernon (1913) and others found that the activation of pancreatic juice was catalytic and considered enterokinase an enzyme. Hamburger and Hekma (1902), Dastre and Stassano (1904) and Waldschmidt-Leitz (1923), on the other hand, thought that the activation process was stoichiometric and therefore believed that enterokinase was a sort of co-factor, which formed an addition compound with the inactive zymogen. Much of this controversy was due to crude methodology and to incomplete knowledge on important points: for instance, it was not known that more than one proteolytic activity is present in pancreatic juice nor was it known that trypsin itself can activate trypsinogen. Moreover, the meaning of the word enzyme then was not the same as it is today.

In the 1930s, our knowledge of the pancreatic proteases and their activation was greatly increased by the work of Kunitz and Northrop (1935, 1936).

They isolated and crystallized chymotrypsinogen and chymotrypsin, trypsinogen and trypsin, plus a trypsin inhibitor present in pancreatic extracts. They demonstrated the autocatalytic activation of trypsin and the ability of trypsin (but the inability of enterokinase) to activate chymotrypsinogen. Finally, Kunitz (1939 a) partially purified enterokinase from swine duodenal contents and used the preparation for important experiments on the mechanism of formation of trypsin from trypsinogen by means of enterokinase. He definitely established that this process is truly enzymic in the modern sense of the word.

In 1956, Yamashina improved Kunitz's (1939 b) purification procedure, and obtained a much purer enterokinase preparation. The total carbohydrate content was remarkably high, nearly 30 %. The sugars and aminosugars present were fucose, mannose, galactose, glucosamine, and galactosamine. It was concluded that the enterokinase is a glycoprotein, as previously suggested by Kunitz.

The general concept when my studies were started was that enterokinase is secreted from the intestinal wall with the intestinal juice, and that the activation of trypsinogen takes place in the intestinal lumen. Pavlov (1907) in his Nobel Prize lecture in 1904 stated that enterokinase was "dank der Reizwirkung des Pankreaseiweissfermentes sezerniert." Florey et al. (1941) concluded from an extensive survey of the literature on the secretions of the intestine up to that time that the balance of evidence was that juice obtained by experimental methods from the jejunum and ileum is an alkaline fluid which, like that from the duodenum, contains only two secreted digestive enzymes - amylase and enterokinase. Also in the small number of more recent studies on enterokinase (see, e.g., Hadorn et al., 1969), as well as in modern textbooks (see, e.g., Samson Wright's Applied Physiology, 1971, p. 409, or Lehninger's Biochemistry, 1971, p. 434), the concept of enterokinase as a secretion product prevails. The concept seems to have been mainly based on the finding that the intestinal juice contains enterokinase activity. In this connexion it is noteworthy that Kunitz (1939 b) and Yamashina (1956 a) used the secretion present in the duodenums of pigs after slaughter as a starting material for the purification of enterokinase. It was also early observed that the enterokinase activity is high in the proximal part of the small intestine. Partly therefore the Brunner glands in the submucosal tissue of upper duodenum have been suggested as the most probable site of formation and secretion of enterokinase. The crypts of Lieberkühn have been regarded as an alternative place of origin (see, e.g., Harper's Review of Physiological Chemistry, 1971, p. 219.). However, no evidence based on direct localization experiments had been provided in favour of the first site or the other.

The present study will contain the following sections.

1. Quantitative distribution of enzymes in different parts of the villi and crypts
2. Determination of enterokinase activity

3. Anatomical localization of enterokinase
4. Site of the physiological function of enterokinase

QUANTITATIVE DISTRIBUTION OF ENZYMES IN DIFFERENT PARTS OF THE VILLI AND CRYPTS

Method for isolation of different parts of the villi and crypts

A technique was developed that includes cutting frozen pieces of intestinal wall in a cryostat in such a way that serial sections of only cross-sectioned villi or crypts are obtained.

A small piece of rat jejunum was cut out. After removal of the mesenterium, it was opened along the insertion line of the mesentery. The mucosal surface was cautiously blotted with soft paper. Alternatively, the opened intestine was rinsed by immersion in cold saline solution. The piece of intestine, villi pointing upward, was spread over a rectangular hole, 6 mm wide and 1-2 cm long, cut in a piece of suitable supporting material, e.g., chromatography paper (Whatman No 1) or filter paper. The edges of the moist tissue adhered to the support, and a even surface of serosa could be obtained. Care had to be taken not to stretch the tissue.

In a cryostat at -18°C , the intestinal segment was then applied with its serosal side to a 4-5 mm frozen rectangular block of 0.1 % agar in water that was attached to a cryostat chuck. The agar-ice block had previously been cut with the microtome to provide a smooth supporting surface parallel with the cutting plane of the knife. The tissue adhered immediately to the agar-ice block and the whole piece of intestine was frozen within 1 min. The edges of the tissue were trimmed with a razor blade to give a tissue block with a surface of about 4 x 8 mm (adult rat jejunum). For each experiment, many tissue blocks were prepared in order to allow a choice of the most suitable one, i.e. the one that was the most even and had maximum thickness. The tissue blocks were inspected under a magnifying glass to ascertain that the villi had not been bent during the preparation.

Cryostat cutting was performed at -18°C with the micrometer set at $10\ \mu$ ($20\ \mu$ in some experiments). The sections were combined in pairs or collected in groups of somewhat greater number and put into small homogenization tubes. Single sections for histologic examination were taken before and after each collection. They were attached to microscope slides for immediate inspection under a phase contrast microscope. After staining with methylene blue, the sections were later examined again with the light microscope. The homogenization tubes were chilled with crushed ice, and the sections were homogenized in a small volume of water or sodium chloride with the aid of a small homogenizer of the Potter-Elvehjem type (1936). The homogenates were used for enzyme assays and determination of nitrogen (protein) without previous centrifugation.

The most critical point of the procedure is the attachment of the intestinal segment to the ice block. The aim is to get a tissue block as thick and even as possible. Subsequent cutting then gives a larger total number of sections, fewer tip sections with only a part of all tips cut, and a shorter transitional zone with sections containing both villi and crypts (thus a sharper limit between the bases of villi and crypts); therefore a better separation of the different parts of the villi and crypts. The tissue blocks most suitable for cutting were obtained from adult proximal rat jejunum. For anatomical and technical reasons, these became much more thick and even than pieces from adult ileum or from the delicate intestine of suckling rat. Rat villi are very suitable for cutting because they stand in good order and are so uniform, stable, and regular. The less uniform human villi make human intestine more difficult to work with. Another disadvantage with human intestine is that the thick muscle-layer and the serosa have to be removed before attachment to the ice block. This means that the human intestinal pieces have to be frozen onto the agar-ice with the trimmed surface of the sub-mucosa, which usually does not give the same even attachment as when the smooth serosa of rat intestine is applied.

Cutting series were not analysed for enzyme activities if microscopic examination revealed that they were not sufficiently successful. Usually only one out of five series was finished for this reason.

Morphological observations

Morphological examination of the sections revealed that all tips were never hit simultaneously by the knife, partly because the villi vary somewhat in length and partly because it is impossible to obtain an absolutely even attachment of the intestine to the ice block (the total thickness of the tissue block of adult rat jejunum is only in the order of 1 mm and in suckling rat it is much less). For similar reasons, the limit between the villi bases and the crypts was also somewhat diffuse, and there are always a number of sections that contain both the basal parts of the villi and the superficial parts of the crypts. This led us to define the extension of the "average villus", which thus represents the mean of several hundreds of villi (see Fig. 1).

Each cross-sectioned villus is composed of a single layer of epithelial cells arranged around an inner core of lamina propria. The shape is characteristically ovale in rat and is similar along the whole length of the villi, except for the tips which are more circular in cross-section. The uniformity is especially well expressed in sections from proximal jejunum. Human villi are less uniform, but are usually rather circular in cross-section in jejunum and ovale in duodenum. The cross-sectioned crypt is composed of a single layer of cells, pyramidal in shape, arranged around a central lumen which gives the whole structure a circular appearance. Lamina propria is seen between the crypts. The crypts are much more numerous than the villi and several crypts open their mouths at one villus base.

Localization of disaccharidases, dipeptidases, and alkaline phosphatase in rat

In paper I, a quantitative comparison of the distribution of alkaline phosphatase, disaccharidase (maltase, sucrase, isomaltase, trehalase, lactase, cellobiase), and dipeptidase (L-alanyl-L-proline dipeptidase, L-alanyl-L-glutamic acid dipeptidase, and glycyl-L-leucine dipeptidase) activities was performed (Fig. 1).

The alkaline phosphatase activity was found to have a distinct apical localization in the villi. When the specific activity was calculated, the maximum was located very close to the tips of the villi, and the activity decreased sharply toward the bases. A similar curve was obtained when the total activity was calculated. A decrease in the tip region was seen only in the curve of total activity, and it reflects the smaller amounts of tissue in the superficial sections. In the most basal parts of the villi, comparatively very weak activity was found. In the crypts, no or negligible activity was found.

The quantitative localization of the maltase and sucrase activities was not identical with that of alkaline phosphatase. Their distribution curves were rather similar, with high activity along a considerable length of the villi. When the total activity was calculated, the maximum was located approximately in the middle of the villi, or somewhat on the apical side of the middle. When the specific activity was calculated, the apical localization was more marked. In both cases, the activity was lower in the parts very close to the tips. Thus, the decrease is not only due to smaller amounts of tissue in the most apical part of the villi, but is also caused by a lower enzyme content in the epithelial cells located there. In the basal parts of the villi, the activity also decreased; in the crypts, there was no or negligible activity. The decrease of activity in the region with sections containing both villi and crypts seems partly to be an effect of the gradual replacement of the villi with crypts, but the curves also indicate that the disaccharidase activities are lower in the villous epithelium close to the bases of the villi. Also isomaltase, trehalase, lactase, and cellobiase activities were found to have the same principal distribution profile as maltase and sucrase.

The L-alanyl-L-proline dipeptidase activity had a distribution that was rather similar to that of the maltase and sucrase activities. Both when the total and specific activities were calculated, there was a considerable activity over most of the villi range. L-alanyl-L-glutamic acid dipeptidase and glycyl-L-leucine dipeptidase activities had similar profiles as L-alanyl-L-proline dipeptidase.

Localization of β -galactosidases and acid phosphatase in rat

In paper II, a quantitative comparison of the distribution of the two rat intestinal β -galactosidases, neutral and acid β -galactosidase, and the acid phosphatase was performed. Distribution curves were determined

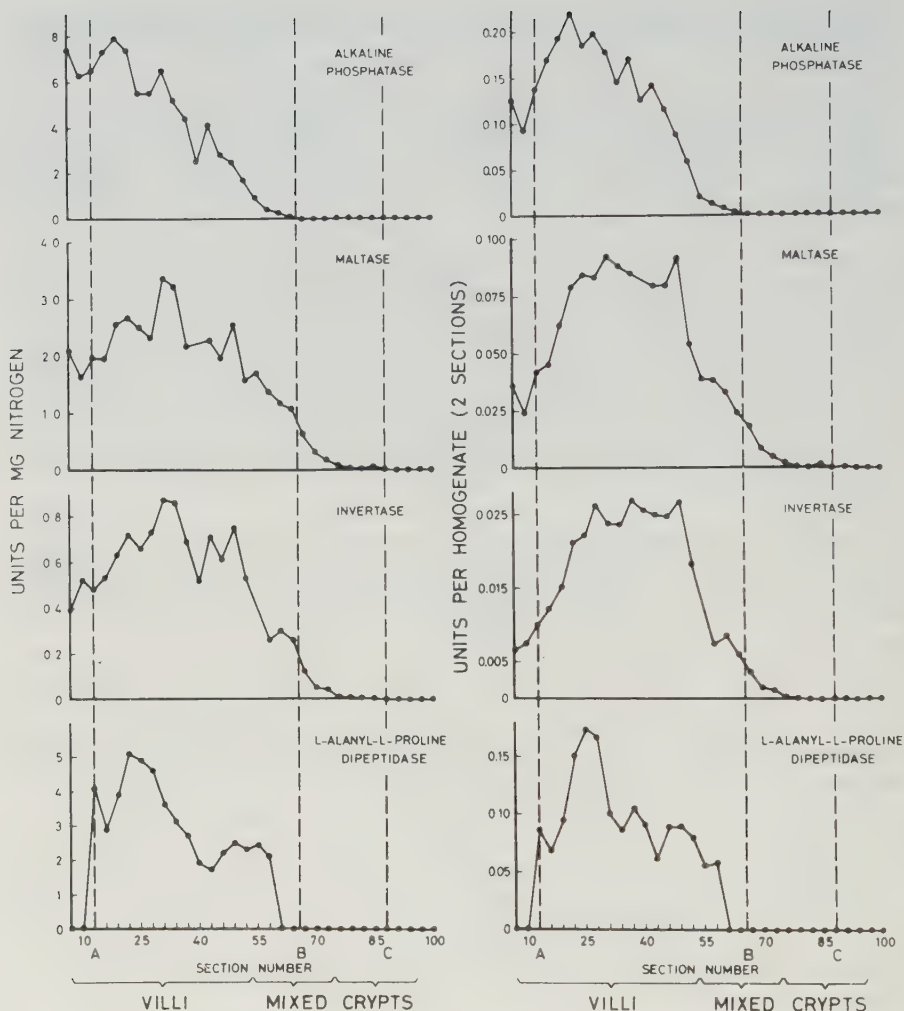


Fig. 1. Quantitative distribution of alkaline phosphatase, maltase, sucrase (invertase), and L-alanyl-L-proline dipeptidase activities along the villi and crypts of rat jejunum. Every third serial section, $10\ \mu$ thick, was taken for histological examination and the intermediate sections were combined in pairs, homogenized and analysed. A = 50 % of the villi cut in the tips; B = 50 % of the villi replaced by crypts; C = 50 % of the crypts replaced by submucosal tissue (from paper I).

and compared for all three enzymes in the jejunum and ileum from both adult and suckling rats (12 days old), because previous studies had shown that the activities of the two β -galactosidases differ in the jejunum and the ileum of adult rats and also change during postnatal life (Asp *et al.*, 1968;

Koldovský & Chytil, 1965; Koldovský *et al.*, 1966).

The neutral β -galactosidase, i.e. brush-border lactase, always decreased strikingly from the mucosal towards the serosal side of the intestinal wall. The enzyme seemed to be located exclusively in the villi in all of the kinds of intestinal preparations studied. The highest activities were present in the apical halves of the villi. The distribution profile is thus the typical one for a digestion enzyme. The distribution of the acid β -galactosidase activity differed markedly from that of the neutral β -galactosidase. The profiles were rather flat with specific activities of the same order in both villi and crypts. An interesting exception was found in the ileum of suckling rats, where the high acid β -galactosidase activity, similar to the neutral enzyme in all experiments, was located mainly in the villi. Acid β -galactosidase also showed a very steep decrease in activity towards the crypts. Also the profiles for the acid phosphatase were rather flat in all kinds of intestine except in the ileum of suckling rat, where the profile showed a downhill gradient from the mucosal to the serosal side.

Paper I and II thus showed two principally different distribution profiles: one digestion profile in common for digestive enzymes, and one flat profile for lysosomal enzyme activities such as acid phosphatase and acid β -galactosidase. A third different profile is given in Fig. 2, which shows the localization of thymidine kinase, an enzyme related to cellular proliferation. It catalyzes the first step of a pathway leading to thymidine triphosphate and thus contributes one of the precursors for DNA synthesis. Thymidine kinase is predominantly found in the crypts and decreases rapidly as the cells reach the crypt-villus junction. The sudden decline has been attributed to a short half-life of this enzyme in rat intestine (Imondi *et al.*, 1969).

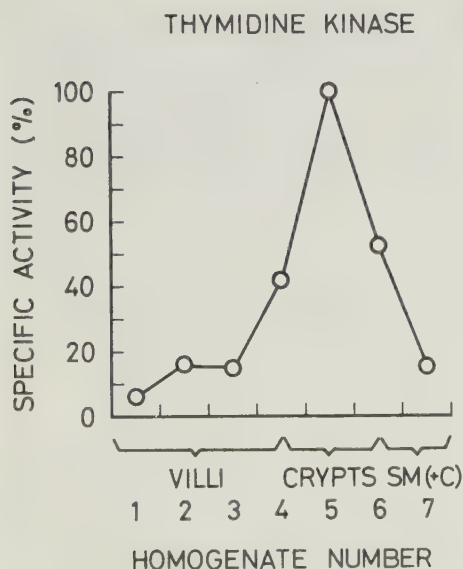


Fig. 2. Quantitative localization of thymidine kinase within the wall of rat jejunum. The last homogenate contains sub-mucosa (SM) but also a slight amount of crypt tissue (C). The highest specific activity (units/gram protein) was arbitrarily set at 100, to obtain relative activities (unpublished work).

Localization of enzymes in man

In paper III, the cryostat sectioning technique was applied to human small intestine. The quantitative distribution of sucrase, maltase, isomaltase, trehalase, lactase (also in a patient with hypolactasia), leucyl naphthylamidase, alkaline and acid phosphatase, acid and hetero β -galactosidase, β -glucuronidase, and thymidine kinase along the villi and crypt was studied.

Similar to the distribution in rat, enzymes related to terminal digestion were almost exclusively present along the villi with highest activities in the mid-villi or apical halves of the villi (Fig. 3). In further agreement with the findings in rat, lysosomal enzymes were found to have a rather flat distribution profile. Highest activities of thymidine kinase were found in the crypts, but there was no pronounced decrease in the villi as found for this enzyme in rat. The difference between the two species might be due to a considerably longer half-life for the human thymidine kinase than for the corresponding enzyme in the rat.

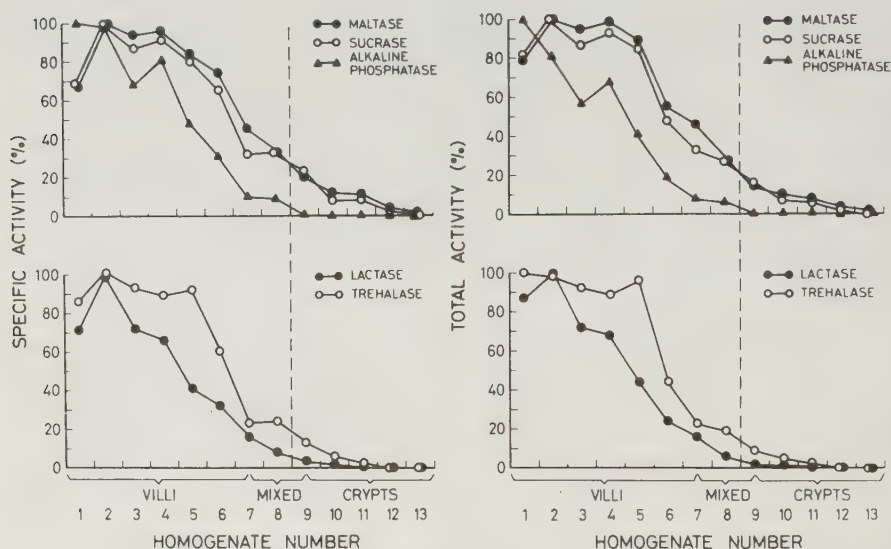


Fig. 3. Quantitative distribution of maltase, sucrase, trehalase, lactase, and alkaline phosphatase activities within the wall of human jejunum. The vertical broken line = about 50 % of the villi replaced by crypts (from paper III).

Within the villi, certain digestive enzymes showed small, but none the less distinct, differences in their distribution patterns. For instance, alkaline phosphatase and lactase were more apically localized than the sucrase and maltase activities (Fig. 3). Furthermore, the distribution of leucyl naphthylamidase was not identical to that of any other brush-border enzymes. In

addition, the villous enzymes showed a general tendency to a more apical localization in the jejunal villi than in duodenal villi. Especially in duodenum, the disaccharidase content of the tip cells was distinctly lower than in cells located in the midvillous region.

All three β -galactosidase activities present in the human small-intestinal mucosa were found to have different distribution patterns reflecting their different functional significance. Brush-border lactase was almost completely localized to the villi. In contrast, acid β -galactosidase and hetero β -galactosidase were found in both the villi and the crypts. The lysosomal acid β -galactosidase had a rather flat distribution profile, whereas the cytoplasmic hetero β -galactosidase activity always had a maximum in the basal parts of the villi and decreased down to about 50 %, in both the crypts and the apical parts of the villi.

The small-intestinal mucosa of individuals with acquired hypolactasia has a low residual lactase activity exerted partly by the acid β -galactosidase, and partly by remaining, and presumably structurally normal, brush-border lactase (Asp, 1971). The distribution profile for the residual brush-border lactase appeared to be the same as in intestine with normal lactase activity. Thus a slow synthesis of brush-border lactase seemed to begin in the same phase of differentiation of the villous epithelial cells as in individuals with high lactase activity. There was also a "normal accumulation" of brush-border lactase during the migration of epithelial cells towards the apical parts of the villi, which lends no support for an increased rate of degradation of the enzyme in individuals with hypolactasia.

Histochemical staining methods have given valuable information about the functional organization of the intestinal mucosa, including the localization of different enzymes. However, such methods are limited and not free from objections. Quantitative studies concerning the distribution of enzymes in the different parts of the villi and crypts are therefore of great interest. The main advantages of the present micro-dissection technique over the staining methods are:

1. it allows an accurate quantitative determination of the enzyme activity at a certain level, whereas the amount of colour obtained in a histochemical staining for an enzyme may be dependent not only on the total enzyme activity present, but also on other factors, e.g., damage of the membranes, which may increase the penetration of the substrate and staining reagents (Dawson & Pryse-Davies, 1963);
2. it allows a direct comparison in the same preparation of two or several enzyme activities section by section;
3. difficulties in obtaining good specific staining methods for certain enzymes are avoided.

The principal of the preparation technique with serial tangential sections of gastrointestinal tissue was utilized a long time ago by Holter and Linder-

ström-Lang (1934), in studies on the mucosa of the stomach, and by van Genderen and Engel (1938), in studies of the distribution of amylase, maltase, and glycylglycine dipeptidase activities in small-intestinal mucosa of rats. van Genderen and Engel could not carry out histological observations and enzyme analysis on alternating sections from the same series, but used separate section series of fixed tissue for histological identification. Such a procedure involves considerable errors. The authors also mention that it was impossible to clearly delimit the different layers, because the limits vary in different pieces and the tissue also shrinks in fixing. Furthermore, different enzyme activities were analysed in different section series. In the present technique, we have avoided these disadvantages. That there is only partial agreement between the present results and those of van Genderen and Engel regarding maltase and dipeptidase is perhaps due to differences in preparation technique, but also to the lack of sufficiently sensitive and reliable enzymatic microassays at that time. van Genderen and Engel usually found maximum amylase activities in the apical parts of the villi, but sometimes there was an expressed maximum in the Brunner glands and in the muscle layer. Their results concerning the maltase activity agree with our results, although they found a maximum in the transitional zone in fasting animals. Especially high dipeptidase activity was found at the level of the Paneth cells and in the Brunner glands, which is inconsistent with our findings.

Digestive enzymes (different disaccharidases, alkaline phosphatase and leucyl naphthylamidase) had the same principal distribution profile, i.e. practically all the activity in the villi and only slight activity in the crypts. As all these enzymes are located in the superficial brush-border region of the epithelial cells (Crane, 1968), it seems reasonable that the general shape of the distribution curves with gradually increasing activities along the villi, at least partly, is a functional expression for the gradual morphological development of the brush border. This differentiation is one important feature in the general development of the cell, and it results in an elongation and narrowing of the microvilli so that the free digestion-active surface of the apical cell becomes much larger than that of a more basically located cell (Brown, 1962).

Similarly, the quantitative localization of absorptive functions such as the uptake of sitosterol and cholesterol may be correlated with the morphological development of the cells, especially the surface area (Fig. 4). But the specificity mechanism involved in sterol absorption (cholesterol is absorbed ten times more than sitosterol (Sylvén & Borgström, 1969)) seems to be well developed already in young absorptive cells, because the ratio between absorbed sitosterol and cholesterol was always constant in different parts of the villi and crypts (Sylvén & Nordström, 1970).

Although the digestive enzymes had the same principal distribution profile, certain of them displayed interesting differences in their quantitative distribution on the villi. For instance, alkaline phosphatase was more apically

localized than sucrase and maltase activities both in man and in rat, and also a greater part of the total activity of human lactase than of sucrase and maltase was present in cells of relatively high age. These differences

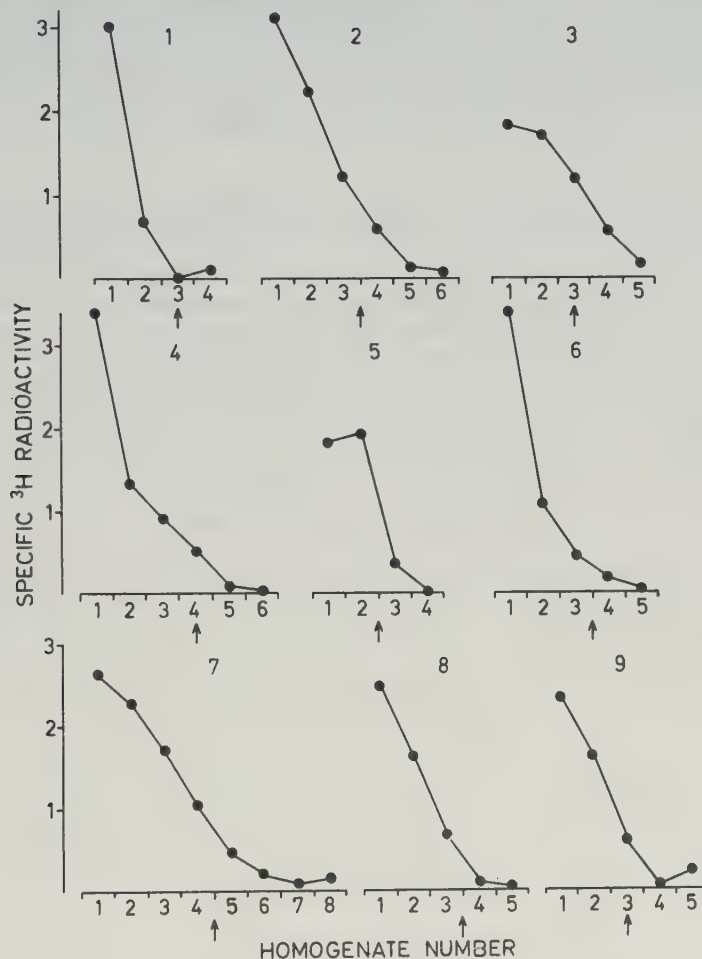


Fig. 4. Localization of sitosterol-22,23-³H uptake in different parts of the villi and crypts after intraduodenal administration of a sitosterol-22,23-³H tracer in micellar solution. The radioactivity in each homogenate is expressed as the specific radioactivity, i.e. as the fraction of total radioactivity in the tissue sample divided with the fraction of total nitrogen content in the tissue sample. The arrows indicate the approximate limit between villi (left of the arrow) and crypts (right of the arrow). Samples 1, 2, 4, 5, 7, and 8 were taken at approximately 20 per cent length and samples 3, 6, and 9 at approximately 50 per cent length from the proximal end of the small intestine. Similar curves were obtained with cholesterol. (From Sylvén & Nordström, 1970).

indicate that the dynamics for different functional properties of the brush-border are not identical. It is further possible that such differences, at least partly, reflect different changes in the ultrastructure of the brush-border membrane that might occur during the migration of the cell along the villi. This concept receives some support from the results reported by Eichholz (1968). He found that sucrase, maltase, and isomaltase activities shared a localization in the same membrane particle, which could be released from the membrane by papain treatment leaving behind trehalase and alkaline phosphatase activities. Similar results are obtained with isolated rat brush borders (see paper VI).

Rey *et al.* (1971) have recently suggested that an increased rate of cellular migration might cause depressed levels of brush-border enzyme activities. Findings showing more expressed depression of lactase activity than of α -glucosidases, such as sucrase and maltase, were considered to be consistent with this hypothesis, provided that protein synthesis begins later for lactase than for α -glucosidases during cell migration toward the tip of the villi. Furthermore, the persistent decrease of lactase activity in a histologically normal intestinal biopsy specimen of many gluten-free coeliac patients would only, in this case, bear proof of a younger than normal cell population. The increased RNA content in epithelial cells of such patients was taken as further evidence for the view (Frič *et al.*, 1969). The present study (III) seems to give experimental support to the hypotheses of Rey *et al.* Lactase has been found to have a more apical localization than sucrase and maltase in direct comparison, which indicates that the level of lactase activity would become easier and more strongly depressed if the cells did not reach their normal age before desquamation. However, if the hypotheses are correct, one can expect also a persistent decrease of alkaline phosphatase in the patients with persistent decrease of lactase, alkaline phosphatase being located at least as apically as lactase.

Acid β -galactosidase activity increases five-fold in specific activity in rat ileum from birth to the 10th day of life, whereas in the jejunum this enzyme gradually decreases to the adult level. Neutral β -galactosidase is highest at birth along the whole rat small intestine; it then gradually decreases to a low adult level (Asp, 1971). Acid β -galactosidase had a diverging distribution profile in the ileum of suckling rat, i.e. a digestion profile, and also acid phosphatase showed a downhill gradient from the mucosa to the serosal side. These results indicated the possible involvement of lysosomal structures in the digestion during the postnatal period. It is of interest to correlate these findings with a recent cytological study of intestinal absorption in the suckling rat by Cornell and Padycula (1969). They found that soon after the initial ingestion of milk, a large, yellow, membrane-limited, protein-body appeared in the supranuclear region of ileal absorptive cells; also many membrane-limited vacuoles arose between this body and the microvilli. Exogenous protein (and other material) was found to become segregated in these membrane-limited vacuoles and then appeared in the supranuclear body. The body and the vacuoles were found to be rich in certain hydrolytic enzymes suggesting the presence of a highly developed lysosomal system. On about the 18th postnatal day, the surface membrane of the ileal absorptive cells becomes impermeable to

macro-molecules, i.e. an adult type of cell appears (Clarke & Hardy, 1969); the supranuclear body and associated structures disappear; the strikingly high activities of acid β -galactosidase and certain other enzymes decrease to adult levels, but brush-border enzymes, such as sucrase, increase (Heringová *et al.*, 1965; Baintner & Veress, 1970). This is also the time of weaning. Concerning the possible substrate for acid β -galactosidase, it has been suggested that this enzyme can help in hydrolyzing dietary lactose during the postnatal period (Asp, 1971).

Our technique for isolation of different parts of the villi and crypts has been adopted in several other laboratories. Some of the publications that have appeared are:

- a) Moog and Grey (1967, 1968) found a distinct apical localization of alkaline phosphatase activity in the intestinal villi of the mouse, which agrees with our findings in rat and man. Using two different substrates (phenyl phosphate and β -glycerophosphate) they could further demonstrate a different activity ratio for the two substrates in the tips and in the bases of villi, which indicates a different localization of two alkaline phosphatase isoenzymes present. It was hypothesized that one molecular form of phosphatase is converted to another as the cells glide up the villi, i.e. a sort of molecular differentiation.
- b) Grey and LeCount (1970) found differences between the distribution of alkaline phosphatase and leucyl naphthylamidase along the duodenal villi of the chick, alkaline phosphatase having a more apical localization.
- c) Das and Gray (1969) studied the incorporation of thymidine- ^3H and leucine- ^{14}C in different parts of the villi and crypts of the rat to correlate directly the site of DNA and protein synthesis in small intestine. Whereas DNA synthesis was maximum in the lower crypt and abruptly decreased in upper crypt and lower villus, protein synthesis continued in the stage of cell maturation when, for instance, the sucrase activity increased steeply. The authors found the micro-dissection technique to be very reproducible.
- d) Herbst and co-workers (1970) investigated the relation of pyrimidine biosynthetic enzymes to cellular proliferation in rat intestine during development. The activities of aspartate transcarbamylase and dihydro-orotase, two enzymes of the *de novo* pathway, were greatest in crypts, and decreased gradually along the length of villi to about one-quarter to one-third of that detected in crypts. Thymidine kinase and uridine kinase, two enzymes of the salvage pathway, also showed maximum activities in the crypts. Uridine kinase decreased slowly to low levels in the apical villus, whereas thymidine kinase declined dramatically to negligible levels along the whole length of the villus. The pyrimidine biosynthetic enzymes, which are involved in the DNA and RNA synthesis and are consequently related directly to cell division, are thus predominantly present in the proliferative zone of the intestinal epithelium. Elevated activities of the pyrimidine biosynthetic enzymes could also be correlated with active cellular proliferation.

- e) It has been suggested that enzymatic determinations of enzymes involved in cellular proliferation, especially thymidine kinase, together with analyses of enzymes related to cellular differentiation, such as disaccharidases, alkaline phosphatase, and dipeptidases (I, III), and lysosomal enzymes (II), can be used to distinguish the crypt from the villus during healthy or diseased states (Fortin-Magana *et al.*, 1970). It must be noted, however, that more than negligible activity of thymidine kinase is present along the villi in the human gut (III).
- f) Ugolev and co-workers (1970) studied the distribution of sucrase and dipeptidase activities along the villi and crypts of the rat and chick. The results were discussed in relation to Ugolev's concept of membrane digestion and various transport processes.
- g) Herbst and Koldovský (1972) studied the cell migration and cortisone induction of sucrase activity in rat small intestine. Comparison of villi and crypts at various times after cortisone treatment showed that the leading edge of sucrase activity proceeds toward the tip of the villi at the same rate as the advancing edge of newly formed cells.
- h) Alpers (1972) recently demonstrated a general correlation between relative degradation rate and size of intestinal brush-border proteins, a faster turnover being found for larger and presumably more superficially located membrane proteins. The micro-dissection technique was used in control experiments, which were important for the interpretation of the results.

A few studies concerning the quantitative distribution of enzymes along the villi and crypts have also been performed with other methods. This makes it interesting to compare the results:

Webster and Harrison (1969) were able to obtain a fractionated removal of the epithelial cells from the tips to the bases of the villi by EDTA-treatment and intense vibration of the whole everted rat intestine. They reported a general agreement between their results and those of Dahlqvist and Nordström (1966) and of paper I regarding the pattern of development of alkaline phosphatase and sucrase. Webster and Harrison's method produces relatively large amounts of fresh material, but it is obviously much less exact than, and not as well-controlled as, the sample collection with the micro-dissection technique. Moreover, the whole crypts are harvested as a single fraction.

Imondi and co-workers (1969) designed an "intestinal planing apparatus" for fresh rat intestine. Its operation allowed successive cuttings of 125 μ of the villi. Subsequently, fractions referred to as "upper crypt" and "lower crypt" were obtained by gentle and heavier scraping of the intestinal wall with a glass slide. This too is a method that does not permit the same high degree of precision, resolution, and control in the separation of cells of different location as the cryostat sectioning technique. Imondi *et al.* studied several enzymes involved in the metabolism of nucleic acid pre-

cursors. Some of the enzymes increased along the villus, which was thought to provide the animal with a mechanism for salvaging those nucleic acid precursors that might otherwise be lost in the excreta. Thymidine kinase was found in young proliferation cells in the crypts with only very little activity in the cells of the villi. The estimate of relative proportions of cell types on the villi from histological sections of cross-sectioned villi revealed 60 % to be epithelial. There was little change in this percentage along the length of the villi.

Galjaard et al. (1970) separated the rat intestinal epithelium from the lamina propria, and subsequently, the crypt epithelium from the villus epithelium by free-hand dissection of freeze-dried longitudinal sections under the dissecting microscope. Although several samples are collected, extreme ultramicro-techniques are needed. In a subsequent paper from the same group (de Both et al., 1972), the epithelium was further fractionated according to the age of the cells. Alkaline phosphatase was found to be almost absent from the crypt cells but increased linearly with the age of the villous cells. This finding agrees perfectly with our previous findings (I), as was also pointed out. Aminopeptidase was also present in the villi, but did not change along the length of the villi; this again is similar to our results for the same enzyme (leucyl naphthylamidase) in man (III). Non-specific esterase activity was low in the proliferating crypt cells, but increased during cell maturation in the upper half of the crypt. A further increase in activity occurred when the cell entered the functional pool of the villus, but during subsequent migration no further change was found. By analysing these and other enzymes in normal and X-irradiated rat intestine, de Both et al. (1972) could show that the process of cell maturation in the crypt is affected by increased proliferative activity, and as a consequence, the activity of a number of enzymes (for instance, aminopeptidase and non-specific esterase) decreases which in turn leads to functional impairment of the intestine. It was suggested that similar mechanisms also apply to certain diseases of the intestine, which is very interesting. But it was not explained why the same group had previously (Galjaard et al., 1970) found no decrease at all in villous enzyme activities such as aminopeptidase following X-irradiation, although the experimental approach seems to have been identical.

DETERMINATION OF ENTEROKINASE ACTIVITY

Assay of enterokinase activity

Enterokinase has a very high specificity for trypsinogen and this physiological substrate is in fact the only one that was known for the enzyme. This means that the determination of enterokinase activity has to be performed in two steps as Kunitz (1939a) first described. Trypsinogen is incubated with the enterokinase-containing solution, and then samples are removed from the activation mixture at suitable time intervals and analysed for the amount of trypsin activity produced. In his method, Kunitz took into account the complications due to the action of trypsin formed, i.e. chiefly autocatalytic activation and formation of inert protein from trypsinogen. Both processes were much reduced by using a low trypsinogen concentration, and the formation of inert protein was further suppressed by activation at pH below 6.0.

The standard assay procedure for determination of rat enterokinase activity described below is a modification of a method used by Hadorn et al. (1969), which in turn is based on the method of Kunitz (1939a).

Activation mixture. 0.1 ml of 0.1 M sodium maleate buffer (pH 6.0), 0.1 ml enterokinase-containing solution, and 1.2 ml distilled water were mixed and pre-warmed at 25°, the incubation temperature. Incubation was started by the addition of 0.1 ml trypsinogen solution (2 mg trypsinogen per ml 0.005 M HCl). The commercial trypsinogen was not dialyzed before use. After a suitable time of activation (not more than 60 min) the determination of the amount of trypsin formed was immediately started by the addition of an aliquot of the activation mixture to a tube with the reagents for the assay of trypsin activity. Different dilutions of each sample were usually analysed to check the proportionality and to obtain an optimum reading value. A blank tube was run with omission of trypsinogen. This tube would also reveal any trypsin accidentally present in the enzyme sample. In separate tubes it was also always checked that the trypsinogen itself was free from trypsin and that no autoactivation of the trypsinogen occurred during the experimental procedure. Analysis of samples from an enterokinase stock solution (a pooled homogenate stored in the freeze-box) was further used to check the reliability of the assay system.

Trypsin determination. The substrate used was benzoyl-DL-arginine p-nitroanilide · HCl, which on hydrolysis gives the yellow p-nitroaniline. Benzoyl-DL-arginine p-nitroanilide · HCl was dissolved in a small volume of dimethylsulfoxide and then diluted with 0.05 M Tris buffer (pH 8.2) containing 0.02 M CaCl₂, to give a substrate concentration of 0.001 M (Erlanger et al. 1961). Incubation at 25° was started by the addition of 0.1 ml activation mixture to 0.5 ml substrate buffer solution,

and stopped after 60 min by the addition of 0.1 ml 30 % (v/v) acetic acid. After centrifugation to remove turbidity, the color was read with a Vita-tron filterphotometer at 409 nm. A standard curve with different amounts of p-nitroaniline was made for the calculation of the enterokinase activity. A unit of trypsin is the activity that hydrolyzes 1 μ mole of benzoyl-DL-arginine p-nitroanilide $\cdot$ HCl per min. 1 unit of enterokinase is the activity that activates 1 unit of trypsin per min.

During the working out of this assay procedure, we studied the influence of enzyme and substrate concentrations, autocatalysis, divalent metal ions, ionic strength and pH to have incubation conditions that, as far as possible, fulfilled the criteria for optimum enterokinase activity. The results of these studies, which had important practical consequences, are given below (see sections Influence of enterokinase and trypsinogen concentrations and Influence of ions).

The above-described assay procedure was the original method used (paper IV). During the course of this study, some modifications have been made. In paper VII, we introduced the use of bile salts as strong activators of the enterokinase activity. 1.2 ml of a 4.1 mM mixture of bile salts (taurocholate-taurodeoxycholate; 2:1, w/w) in 0.01 M sodium maleate, pH 6.0, was added to the activation mixture instead of distilled water. This also made the assay system insensitive to the addition of small, varying amounts of bile salts, as may be present in certain samples. When human enterokinase activity is measured, it is questionable whether bile salts should be used (see below). On the other hand, it has proved necessary to use 80 mM NaCl in the trypsin determination step (increasing the ionic strength) when analysing human enterokinase, or the linearity will be lost.

All samples containing interfering amounts of trypsin (for instance, many samples of the *in vivo* experiments in the rat, paper VII, and also duodenal juice) had to be filtered on Sephadex G-100 (Hadorn *et al.*, 1969) to remove the trypsin before analysis of the enterokinase activity. This was a time-consuming and therefore limiting procedure. Fig. 5 shows a filtration of a human duodenal juice sample with high activity of trypsin. Enterokinase was eluted in the void volume, whereas trypsin elution was considerably delayed. Filtration also removed the yellow bile colour from the enterokinase samples which is necessary.

The assay procedure for enterokinase activity allows determination of the activity present in particle form in crude preparations such as homeogenates. This was the main reason for choosing a colorimetric method for the assay of trypsin. In preliminary experiments, we also tried to use tosyl-L-arginine methyl ester as trypsin substrate and to measure the hydrolysis in a spectrophotometer or in an automatic titration unit (pHstat). The spectrophotometric method was sensitive to the turbidity of the reaction mixture and required precipitation of the protein with ethanol before reading. This, in turn, led to loss in sensitivity and other complications. The pH-stat method was inconvenient and laborious for a large scale analysis.

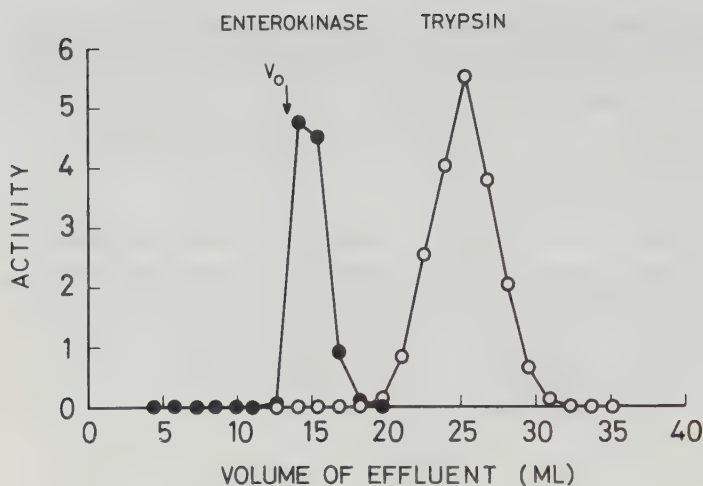


Fig. 5. Separation of enterokinase and trypsin of human duodenal juice by gel filtration on a Sephadex G-100 column according to Hadorn *et al.* (1969). All samples containing endogenous trypsin had to be filtrated before the enterokinase activity could be determined.

The assay of enterokinase with trypsinogen as the substrate is highly sensitive and specific. However, the use of trypsinogen also has clear disadvantages and it would be desirable to have another less complicating substrate. Recently, Maroux *et al.* (1971) studied the specificity of porcine enterokinase using a highly purified preparation and found that enterokinase is active toward benzoyl-L-arginine ethyl ester and tosyl-L-arginine methyl ester. However, these two are typical small synthetic trypsin substrates. The specific activity of trypsin was also found to be as high as, and much higher than, respectively, the corresponding activity of enterokinase. These substrates therefore are not suitable for practical purposes.

When bovine trypsinogen is activated by enterokinase, a Lys-Ile peptide bond in the trypsinogen molecule is split leading to the removal of a hexapeptide Val-Asp₄-Lys (Rovery *et al.*, 1952; Davie & Neurath, 1955; Yamashina, 1956b). Isoleucine becomes the NH₂-terminal amino acid of the trypsin molecule. The chemical events are the same when trypsin activates trypsinogen, but there is an important difference in the specificity as shown by Maroux *et al.* (1971). Enterokinase recognizes not merely the aliphatic carbon chain and the basic amino group of lysine, as does trypsin, but the entire polyaspartyl-lysine structure. The aspartyl residues constitute for enterokinase the signal for a fast and highly specific activation. In contrast, the same aspartyl sequence slows down the action of trypsin, and this effect is thought to give protection against autoactivation in pancreas and pancreas juice (in addition to the trypsin inhibitors present). Consequently, trypsinogen is a much better substrate

for enterokinase than for trypsin. The K_m for the enterokinase-catalyzed activation is 6 times lower and k_{cat} is 2000 times higher (Maroux et al., 1971).

Influence of enterokinase and trypsinogen concentrations

The formation of trypsin from trypsinogen by enterokinase follows the course of a first-order reaction (Kunitz, 1939a). However, in the initial phase of the activation process (corresponding to the conversion of a small part of the total amount of trypsinogen present in the activation mixture) the kinetics are practically of zero order. A linear relation was found between the amount of enterokinase present and the amount of trypsin formed; the time curves too showed linear relations.

The standard concentration of trypsinogen (0.13 mg/ml activation mixture) is clearly below the K_m . However, when higher concentrations of trypsinogen were tried, there was an increasing tendency of the trypsin formed by the action of enterokinase to produce more trypsin by autocatalysis. With the standard conditions used for assay, no important autocatalysis occurred, as concluded from time curve studies and measurements of the "enterokinase" effect exerted by pure bovine trypsin. Lower concentration of trypsinogen than the standard concentration was not desirable because this caused loss in sensitivity. It is important to note that trypsinogen preparations of different purity will give differences in the activation rate with one and the same enterokinase sample. However, in the present study, this has not caused any problem, as all analyses of an experiment have always been performed on one and the same occasion.

Influence of ions

During preliminary experiments on the subcellular localization of rat enterokinase, we observed that EDTA, necessary for the isolation of the brush borders, inhibited the activation of trypsinogen by enterokinase. Recently it was reported that low concentrations of calcium ions activate enterokinase (Milstone et al., 1969; Hadorn, 1969), which suggested that EDTA might inhibit by complex formation with calcium ions. After some preliminary experiments, however, it became clear that a more systematic investigation of the effect of ions had to be undertaken to find the optimum ionic conditions for the assay of enterokinase activity to be used in the localization experiments. Practically no information was available in the literature concerning the influence of metal ions and ionic strength, although the existence of important effects could almost be predicted because of the nature of the enzyme and the substrate.

The effect of various bivalent metal ions (Co^{2+} , Zn^{2+} , Mg^{2+} , Ca^{2+} , Ba^{2+} , and Mn^{2+}) on the activation of trypsinogen by rat enterokinase was studied. Concentrations of metal salts were made 1 mM in the activation mixture. A brush-border preparation was used as source of enterokinase. Co^{2+} had no effect, and Zn^{2+} had a very small negative effect. All other metal ions

tested, including Ca^{2+} , inhibited the enterokinase activity to a considerable extent (reduction to 40-50 % of control value). A more pronounced inhibition was seen at higher concentrations of Ca^{2+} . The inhibition really took place in the activation (enterokinase) step and not in the trypsin determination step, because in separate experiments, it was checked that the assay of trypsin was not influenced by the amount of metal ions transferred from the activation mixture with the sample used for trypsin determination. The lack of inhibitory effect on trypsin could also be predicted, at least for calcium ions, because these are known to activate trypsin (the trypsin assay system has a concentration of around 0.017 M with respect to calcium ions). Co^{2+} and Zn^{2+} had a slight activating effect in lower concentrations, but Ca^{2+} did not activate significantly at any concentration. Co^{2+} and Zn^{2+} together did not increase the small activating effect more than each of these ions did separately. A stronger activation of enterokinase activity by Co^{2+} and Zn^{2+} was observed when the pH of the activation mixture was increased (pH 6.8, 7.4, 7.8). That this was not due to promotion of autocatalytic transformation of trypsinogen was checked in control experiments, in which crystallized bovine trypsin instead of enterokinase was added at zero time to activation mixtures with different pH.

The above-summarized results are from experiments in which brush-border preparations were used as source of enterokinase. With mucosal homogenates, the results were slightly modified although essentially the same.

MgSO_4 present in the commercial trypsinogen preparations used in the standard assay procedure might modify the effect of other added ions. Therefore, we also tested the effect of divalent metal ions on the enterokinase activity with a MgSO_4 -free trypsinogen. The most striking effect with this dialyzed trypsinogen was that a higher activation rate was obtained, which was due to the removal of inhibitory ions. The effect of the various ions was essentially the same as with the commercial trypsinogen, and again we found no significant activating effect of Ca^{2+} .

The influence of different concentrations of NaCl , KCl , Na_2SO_4 , and sodium EDTA on the rat enterokinase activity was tested with mucosal homogenates and with brush-border preparations as source of enterokinase. Soluble enterokinase activity, which was produced by papain treatment of brush borders and subsequent ultra-centrifugation, was also used. Similar effects were observed with all kinds of enzyme preparations. All the salts depressed the enterokinase activity, the degree of inhibition being proportional to the concentration. The effect, therefore, was not ion-specific but seemed to be due to the increasing density of charge in the activation mixture. The order of increasing inhibition exerted by the different compounds agreed well with the values of ionic strength that could be calculated for each of them. In preliminary experiments, it had been ruled out that the inhibition could be exerted in the trypsin determination step and not in the activation (enterokinase) step.

As the ionic strength of the activation mixture had such a great influence on the enterokinase activity, it could be expected that the determination

of the pH optimum for the activation of trypsinogen by enterokinase would be influenced by the buffer used. The use of a maleate buffer with constant molarity (varying ionic strength) gave a pH optimum at 5.6–5.8. No corresponding optimum was found with a maleate buffer of isoionic strength (varying molarity); instead, there was a broad maximum plateau from around 6.0–6.8. Therefore, the pH optimum found with the non-isoionic buffer was rather the effect of ionic strength than of pH, and this finding further illustrates the great sensitivity of the enterokinase activity to changes in ionic strength.

The activation of trypsinogen by human enterokinase was also greatly influenced by the ionic strength of the activation mixture (Fig. 6).

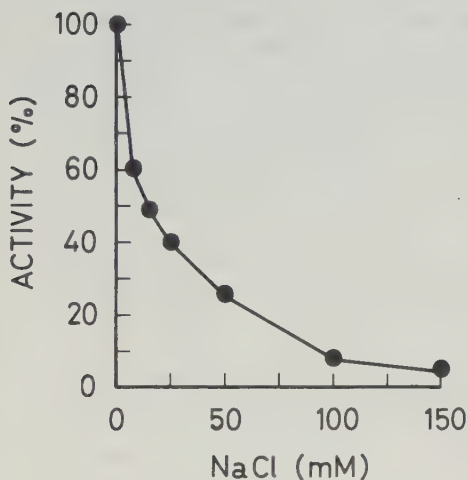


Fig. 6. Influence of the sodium chloride concentration on human enterokinase activity from duodenal juice (unpublished work).

The experiments revealed no important activating effect of metal ions on the activity of rat enterokinase and consequently no Ca^{2+} or other metal ions were added to the activation mixture. High ionic strength in the activation mixture should be avoided because it considerably depresses the activation rate. Furthermore, the ionic strength must be kept constant to allow a correct comparison between different samples.

Although a higher activation rate could be obtained with the same amount of enterokinase if MgSO_4 -free trypsinogen was used instead of commercial trypsinogen, we did not usually dialyze the latter before use. A suitably low ionic strength of the activation mixture could still be achieved. It was also found that the ionic strength could not be reduced indefinitely, as this makes the system increasingly sensitive to very small differences in ionic strength between different samples.

The strong influence of EDTA on the enterokinase activity made it necessary to remove this compound by dialysis before the different preparations of the brush-border isolation experiment could be analysed. If this was not done, erroneous recovery and purification values resulted.

The ionic strength in the activation step is also important when the activity of a highly purified porcine enterokinase is assayed. (Maroux, personal communication).

Influence of bile salts

Hadorn *et al.* (1971) found that the activity of rat enterokinase (isolated brush borders) is about five times increased in the presence of an optimum concentration of bile salts. They initially attributed the increase to the solubilization of membrane-bound enterokinase and suggested that the soluble enzyme was more efficient in activating trypsinogen. In contrast, we found (paper VI) that the increase in activity was due to the ability of bile salts to activate the enterokinase activity irrespective of whether the activity was present in soluble or bound form. Several observations supported this view. First, the solubilization step as such did not seem to cause any significant increase in the activity of enterokinase. Second, the increase in activity was reversible, i.e. depending on the presence of bile salts. Third, the same increase as found with isolated brush borders was also found for both bile-salt solubilized and protease solubilized enterokinase activity; the activity remaining in the sediment (bile-salt solubilization was not 100 %) was also activated. Fourth, certain evidence suggested that the bile-salt solubilization in fact was not a true solubilization, but rather mainly a formation of low-specific-weight aggregates with relatively large membrane fragments.

Fig. 7 shows the activating effect of different concentrations of sodium taurodeoxycholate and taurocholate on the rat enterokinase activity of a mucosal homogenate. An important activation was found both with and without 150 mM NaCl in the activation mixture. When the increase in the reaction rate was expressed as per cent of respective controls (no bile salt added), the maximum degree of activation by taurodeoxycholate at high ionic strength was about twice the maximum activation at low ionic strength. The corresponding difference with sodium taurocholate was much greater. A possible explanation for this phenomenon could be that at low ionic strength the addition of increasing amounts of bile salts also influences negatively through the effect of ionic strength. Part of the explanation might also be that the bile salts in fact are able to counteract the effect of the ionic strength.

It can be calculated that the rate of activation of trypsinogen by enterokinase in the presence of 150 mM NaCl and absence of bile salts is depressed to about 1 % compared with the rate in the absence of NaCl but the presence of optimum concentrations of bile salts. This well illustrates the great sensitivity of the rat enterokinase activity to the ionic strength and bile salts.

The concentration of trypsinogen used in the assay of enterokinase activity is much below the concentration needed to saturate the enzyme. Therefore, it is possible that the bile salts activated by increasing the affinity for the substrate. However, K_m values obtained (at high ionic strength) in the

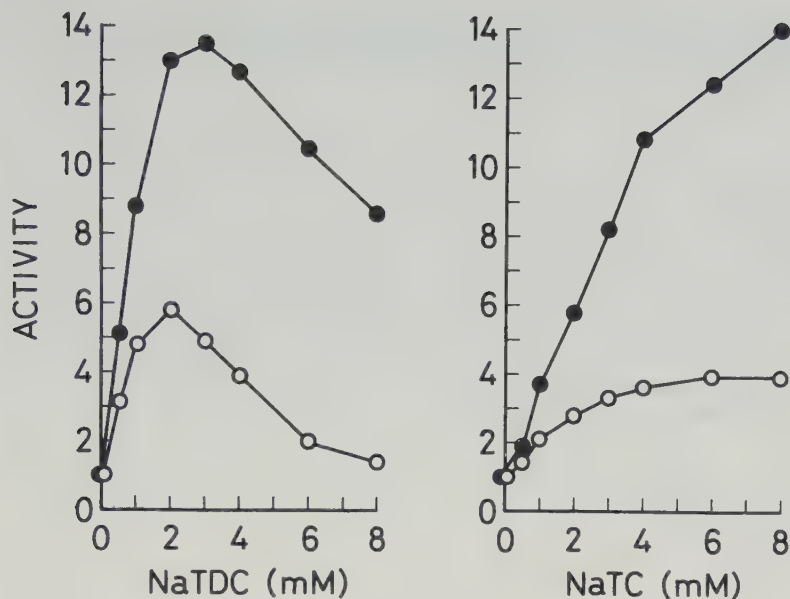


Fig. 7. Influence of bile salts on the rat enterokinase activity in the presence (●—●) and the absence (○—○) of 150 mM NaCl in the activation mixture. NaTDC, sodium taurodeoxycholate; NaTC, sodium taurocholate. The activities in the controls have been arbitrarily set at 1.0 in all experiments and are not equal in absolute numbers (unpublished work).

presence of 1.6 mM taurodeoxycholate or 8.0 mM taurocholate did not differ significantly from K_m -values obtained in the absence of bile salts. The action of bile salts, therefore, seems to be mainly velocity effects. Speculatively, the effect of ionic strength might be on the substrate, the trypsinogen, whereas the bile salts could act on the enzyme or both the enzyme and substrate.

In surprising contrast to the rat enterokinase, human enterokinase activity was found to be only moderately activated (up to 100 %) by bile salts. At least taurocholate also in fact seemed to decrease the accuracy of the assay. Sodium taurodeoxycholate (0–6 mM) and sodium taurocholate (0–8 mM) were tested in the presence and the absence of 150 mM NaCl. Enterokinase in both particular form (mucosal homogenate) and soluble form (duodenal juice which had been filtered on Sephadex G-100) was used.

Finally, it should be mentioned that already Schepowalnikow (1899) observed an increasing activation rate when he added bile to a mixture of pancreatic juice and intestinal juice from the dog!

ANATOMICAL LOCALIZATION OF ENTEROKINASE

Localization along the intestine

High activities of enterokinase were found in rat duodenum, the activity decreasing sharply in distal direction (Fig. 8). But significant activity was still present in the proximal half of jejunum, and some activity was detectable also in distal jejunum and proximal ileum. This distribution pattern was similar to that of the alkaline phosphatase activity, but differed from those of sucrase and trehalase activities.

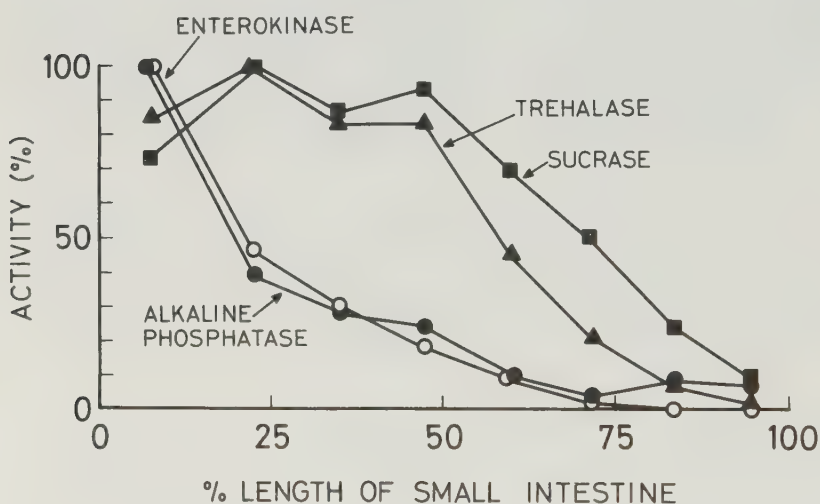


Fig. 8. Distribution of enterokinase and some other enzyme activities along the small intestine from pylorus to distal ileum. The intestine was cut into 6-cm long pieces, rinsed in saline, homogenized *in toto* in water, and analysed for enzyme activities and protein. The cumulative amount of protein in the pieces was used as an expression for the length of the intestine (unpublished work).

In human intestine, we mainly investigated duodenum, where activity seemed to be present in all parts (in collaboration with Å. Nordén).

The concentration of enterokinase to the proximal part of the small intestine was one main reason for using duodenum in the further studies

of enterokinase. The other main reason was that duodenum is the part of intestine to which the trypsinogen of the pancreatic juice is presented.

Localization in the villi and crypts

Quantitative localization of enterokinase in the wall of rat duodenum revealed the unexpected and exciting fact that the enzyme was present in the villi but essentially absent from the crypts (Fig. 9). No detectable activity was present in the submucosal tissue even when the Brunner glands were present. This localization of enterokinase was a regular feature in all parts of duodenum as found in a specially designed experiment in which the localization of enterokinase was studied throughout the entire length of duodenum. This seemed to rule out the possibility that the usually obtained distribution profile for enterokinase could have been created by adsorption secondary to a secretion of the enzyme from deeper layers of the mucosa in some limited part of the duodenum. Thus, the distribution pattern of the enterokinase activity strongly indicated that the digestive-absorptive cells of the villi might be the site of elaboration of enterokinase. Furthermore, the distribution profile was the same as previously found to be typical for enzymes present in the brush-border region of the villous epithelial cells (I, II, III). Consequently, it was logical to suspect that enterokinase could in fact be a surface membrane-bound enzyme. The next step in the investigation was therefore to perform a study on the subcellular localization of the enzyme.

The enterokinase activity, similar to the alkaline phosphatase and trehalase activities, was more apically localized in the villi than sucrase, maltase, and isomaltase activities (Fig. 9). Such differences in the quantitative distribution pattern in the villi have been demonstrated and discussed above (I, II, III). It is noteworthy that the profiles of the disaccharidases in the rat showed differences between duodenum and jejunum similar to those found for the human intestine (III).

The enterokinase localization in human duodenal wall was also studied (paper V; Nordström & Dahlqvist, 1973). As in the rat, the principal localization was in the villi, only small activities being found in the crypts. No activity was detectable in the submucosa containing Brunner glands.

A preliminary note on the localization of rat enterokinase in the wall and within the cell was submitted in 1969 (Nordström & Dahlqvist, 1970).

Subcellular localization

A procedure was worked out for the isolation of the brush borders of the epithelial cells of rat duodenal mucosa. Mucosal scrapings from several animals were homogenized in strongly hypotonic sodium EDTA (pH 7.0) under carefully controlled conditions (Miller & Crane, 1961). After homogenization, intact brush borders were the largest cellular structure present,

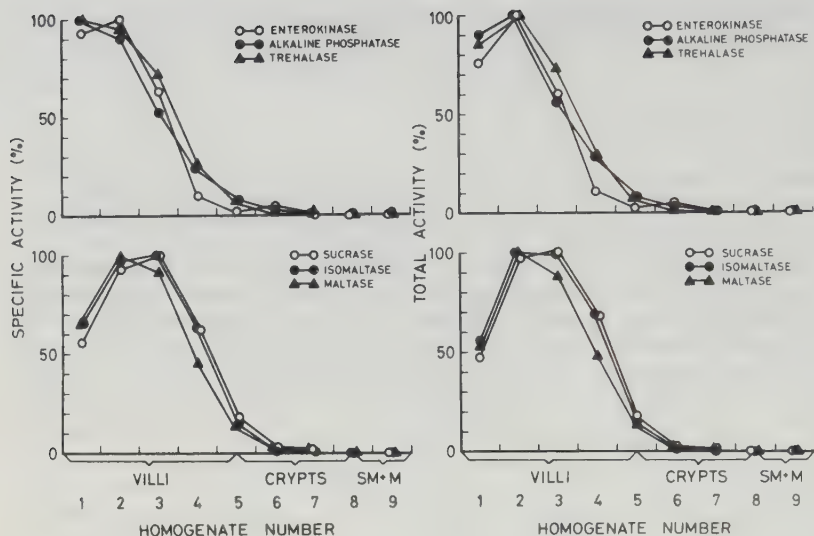


Fig. 9. Quantitative distribution of enterokinase and some other enzyme activities within the wall of the rat duodenum. SM, submucosa; M, muscle tissue. The activity profiles have been put together in two groups according to similarities found in the localization pattern on the villi. (from paper IV).

and it was possible to wash them rather clean by repeated sedimentation (at low speed) and resuspension (crude brush borders). The brush borders were further purified by NaCl treatment which effectively removed present contaminants (mainly nuclei) yielding clean purified brush borders. All steps were checked in the phase contrast microscope.

Enterokinase was found to be present in the brush-border preparations. It was not merely detectable; it was also present and purified to the same extent as sucrase and alkaline phosphatase, two well-known brush-border marker enzymes, both in crude and purified brush borders. Similar results were obtained when the enterokinase activity of purified brush borders was analysed in the presence of the original mucosal homogenate.

The found association of the enterokinase activity with the villous epithelial cells and their brush-border region were obviously surprising and exciting findings in view of the general concept about enterokinase held for many years. They seemed to mean the discovery of a principally new functional property of the brush-border organelle, because enterokinase differed so fundamentally from previously known brush-border enzymes both concerning the type of substrate and the point of action in the sequence of digestive events. The localization findings immediately posed new questions concerning enterokinase and the activation of trypsinogen in the gut. What is the functional localization of enterokinase? What mechanism lies behind

the activity of the enzyme in the duodenal juice?

To obtain information on the morpho-chemical relation between enterokinase and well-known brush-border enzymes, and between enterokinase and the membrane, a study was undertaken on the enzymic release of enterokinase, different disaccharidases, alkaline phosphatase and leucyl naphthylamidase from isolated, duodenal brush borders (VI). Trypsin and chymotrypsin were found to cause a specific, selective release of enterokinase, and papain released enterokinase more easily than it released any other enzyme (Fig. 10). Although trypsin and chymotrypsin did not significantly release any enzyme activities from isolated brush-borders except for enterokinase (chymotrypsin also liberated small amounts of lactase activity), they were also able to release different disaccharidase activities (except trehalase) from the membrane fragments of a mucosal homogenate.

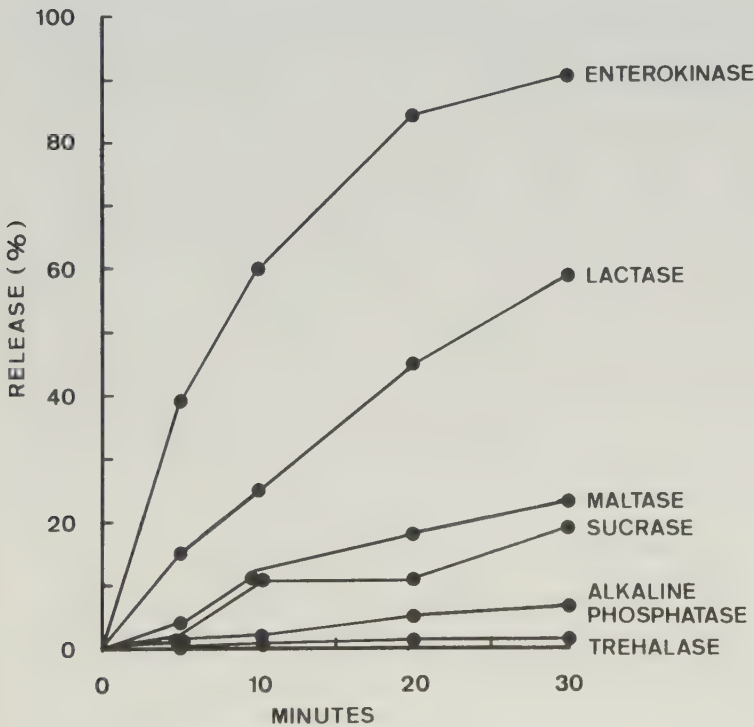


Fig. 10. Release of enzyme activities from isolated rat brush borders by treatment with papain (from Nordström & Dahlqvist, 1971b).

The findings concerning the localization of enterokinase along the rat intestine have recently been confirmed by Lojda and Mališ (1972). Utilizing a slightly modified version of our assay procedure for determination of enterokinase activity, these authors investigated five different animal species and found a proximo-distal gradient in all species, although the decline in enterokinase activity in distal direction was differently expressed in the different species. In the rat, for instance, the enterokinase activity of jejunum was found to be 50 % of the activity in duodenum, whereas the corresponding figure for human intestine was 9 %. In contrast to these results and ours, Eggermont *et al.* (1971) found enterokinase activity only in mucosal biopsy specimens from the proximal part of human duodenum.

Lojda and Mališ (1972) have also confirmed our findings on the localization of the enterokinase activity in different layers of the intestinal wall by means of a histochemical staining method. They could demonstrate the presence of enterokinase activity in the villous epithelium with highest activities in the enterocytes covering the apical parts of the villi. Enterokinase activity was essentially absent from the crypt cells, and no activity was present in the goblet cells, Paneth cells, or Brunner glands. This localization of the enterokinase activity was a regular finding in all five species investigated, including man.

A survey of the oldest literature on enterokinase reveals that all authors did not consider the Brunner glands or the glands of Lieberkühn as the site of origin of enterokinase. Delezenne (1902 a,b) and others reported results suggesting lymphocytes and Peyer's patches as the source of enterokinase. Falloise (1904) and Mellanby and Woolley (1913) reported that enterokinase was present to a greater extent in a superficial layer than in a deep layer of the intestinal wall. However, Falloise found in the same investigation that maltase and amylase were predominantly present in the crypts. Mellanby and Woolley (1913) also found that enterokinase was ubiquitous in the organism and suggested that this provided the organism with a mechanism for the destruction of degenerated tissue, trypsinogen being transported to the particular tissue by the blood.

Our discovery that enterokinase is associated with the brush border of the villous epithelial cells has been confirmed (Holmes & Lobley, 1970; Hadorn *et al.*, 1971). Holmes and Lobley also disrupted purified brush borders from guinea-pig with Tris, separated the resultant microvillous membranes on a glycerol density gradient (Eichholz & Crane, 1965), and found a further increase in specific activity of enterokinase in this membrane preparation over the preparation of purified brush borders.

No studies on the subcellular localization of human enterokinase have been performed, as no method for the isolation of human brush borders has been available. However, the human enterokinase activity present in a mucosal homogenate is bound to particles, as is the case with disaccharidases and alkaline phosphatase (V). Furthermore, human enterokinase has the same principal distribution within the duodenal wall (V) as is found

for enterokinase in the rat (IV) and other species (Lojda & Mališ, 1972). These facts make it highly probable that also human enterokinase is synthesized in the villous epithelial cells and incorporated in the brush-border membrane. Besides, it seems improbable that this enzyme would differ in its cellular and subcellular localization from one species to another. (A direct investigation of the subcellular localization can soon be expected, since a method for isolation of human microvilli is now available (Welsh et al., 1972)).

The enzymic release of enterokinase may have both a chemical and a structural explanation. It is apparent that the linkages of enterokinase to the surface membrane can differ chemically from those of the other enzymes, being more sensitive to the different proteases. It should be noted, however, that the specificities of papain, trypsin and chymotrypsin are not identical. The findings on the release would also accord well with a very superficial location of enterokinase in the surface membrane, possibly even in the glycocalyx. A superficial site in the membrane of this enzyme activity is also indicated by the fact that the proteolytic solubilization of enterokinase of isolated brush borders was not associated with any increase in the efficiency of trypsinogen activation, although this substrate is a large molecule. Furthermore, trypsin and chymotrypsin released almost solely enterokinase from isolated brush borders. But when these proteases were incubated with a homogenate prepared in a way that disintegrates the brush borders into small fragments, they were able to solubilize both enterokinase and a number of disaccharidases. Thus, in the intact brush border, enterokinase is more accessible to the proteases than are the disaccharidases. This indicates ultrastructural differences in the localization between enterokinase and, for instance, sucrase, probably with the site of enterokinase more close to the surface of the membrane.

It has been proposed that the enzyme proteins within the brush-border membrane are not diffusely distributed throughout a protein coat of the membrane but organized in discrete packets, in morpho-chemical subunits as in a mosaic (Crane, 1966; Eichholz, 1968 and 1969). The present results of the enzymic release of enterokinase represent a new indication for the accuracy of this foreseeing concept (cf. Singer & Nicolson, 1972).

The findings concerning the localization of enterokinase would seem to have direct clinical implications: first, an intestinal biopsy from well within the range of duodenum should be included in the analysis program when enterokinase deficiency is suspected (not only juice analysis); second, an analysis of the enterokinase activity should be carried out on whole homogenate, i.e. centrifugation should not be performed before determination; third, the knowledge of the localization may give some guidance in the identification of patient groups who should be investigated concerning a possibly decreased enterokinase activity.

Enterokinase deficiency is a newly-recognized disorder of protein digestion, and six cases are known hitherto. The first cases were described by Hadorn et al. (1969) and Tarlow et al. (1970). The patients are small infants (except

one) and the clinical features are diarrhoea from birth, hypoproteinaemia with hypoproteinaemic oedema, anaemia, and failure to thrive. Their duodenal juice contains essentially no active proteolytic enzymes, but normal activities of trypsin, chymotrypsin and carboxypeptidase appear rapidly if enterokinase is added to the juice. In contrast, the addition of trypsin (bovine crystalline trypsin) causes a very poor activation. Enterokinase deficiency is best treated with a preparation of purified porcine enterokinase or with pancreatic extracts (containing activated proteases) which are taken by mouth (Hadorn, personal communication). Enterokinase deficiency is believed to represent an inborn error of metabolism, because no other abnormalities are detectable in these patients. The subcellular localization of this enzyme then implies that the protein malabsorption in these patients is ultimately caused by the absence of a specific functional protein element of the brush-border membrane. The disorder would in this way be similar to, for instance, the primary disaccharide intolerances (Dahlqvist, 1964).

Intestinal biopsies from patients having pronounced villous atrophy should be expected to have reduced levels of enterokinase activity. Surprisingly, however, the only two investigations in patients with coeliac disease (gluten-induced enteropathy) performed hitherto indicate that the levels are normal or reduced only in few cases, whereas the activities of well-known brush-border enzymes, such as alkaline phosphatase, sucrase, and lactase, are always reduced (Rutgeerts *et al.*, 1972; Woodley & Keane, 1972). Therefore the authors suggested that enterokinase might be synthesized in other cells than the villous epithelial cells and that the presence of enterokinase in the brush borders was an adsorption phenomenon secondary to a secretion of the enzyme. However, because disease of the small intestine produces a marked decrease in the available brush-border surface (including glycocalyx), both constitutive and adsorbed enzymes can be expected to be reduced. Therefore the apparent discrepancy between enterokinase and the other enzymes does not seem to be satisfactorily explained by the idea of adsorption. Adsorption also presupposes a preceding secretion of enterokinase in an inactive precursor form from an unknown site! Furthermore, a secreted enzyme would be expected to be soluble, but enterokinase is not (V). Finally, adsorbed enzymes, such as the pancreatic ones, are known to be removed by the repeated washings and shakings in the procedure for isolation of brush borders (James *et al.*, 1971). It might be pertinent to repeat here that the determination of enterokinase activity involves many problems.

Brunner glands have been suggested as a possible site of origin of enterokinase (Eggermont *et al.*, 1971; Rutgeerts *et al.*, 1972) because enterokinase activity and Brunner glands were found only in biopsy specimens from the upper part of the duodenum. But other studies have shown presence of enterokinase activity in all parts of duodenum (V) and also in jejunum (Lojda & Mališ, 1972). No enterokinase activity is detectable in the Brunner glands. An attempt to find a relation between enterokinase and the Brunner glands have also been made by Goldman *et al.* (1971) who demonstrated an increase in the enterokinase secretion from Brunner's gland pouches in dogs after glucagon stimulation. But the reported results are consistent

with our concept of enterokinase localization. Therefore there is no evidence in favour of Brunner glands as source of enterokinase.

Goblet cells have also been suggested as a possible site of enterokinase formation (Woodley & Keane, 1972). Existing data do not support this view. The goblet cells contain no enterokinase activity, and there is no correlation between the distribution of goblet cells and enterokinase activity along the intestine (Lojda & Mališ, 1972). True, porcine enterokinase has recently been immunochemically localized to the goblet cells of porcine small and large intestine (!) (Takano *et al.*, 1971). However, we have shown that the used antigen preparation was impure; thus no conclusions about the localization of enterokinase can be drawn from their results (V).

Finally, Nasset and Ju (1970) have claimed that enterokinase might be secreted from the crypts. Their work is presented in a short abstract which does not allow a full evaluation but the results do not seem to be very conclusive.

Addendum in the proof: When this treatise is in print, it comes to my knowledge that a study of the enterokinase activity in patients with coeliac disease have also been performed by Lojda and co-workers (Lojda, personal communication). Up to now they have analysed duodenal biopsies from 12 patients with coeliac disease and from 20 patients with other forms of the malabsorption syndrome. A remarkable reduction of enterokinase activity was found in 5 patients with untreated coeliac disease! But there was no disappearance. In the patients with other forms of malabsorption syndrome, a reduction was only found in some patients with milk intolerance (4 out of 9 patients). It is stressed that the activity of enterokinase behaves differently from the activities of other brush-border enzymes.

SITE OF THE PHYSIOLOGICAL FUNCTION OF ENTEROKINASE

No functional or morphological evidence of secretory activity has ever been demonstrated for the mature epithelial cells of the villus. Furthermore, all digestive enzymes present in the brush-border membrane are known to exert their function while still associated with the cells on the villus, and the activity which reaches the intestinal lumen is due to cell shedding in the tips of the villi. Hence, it was obvious that the findings on the cellular and subcellular localization of enterokinase would appear to contradict the current concept of enterokinase as a constituent of intestinal secretion. The results indicated, instead, that enterokinase might be physiologically active in the cells of the villi and that trypsinogen is activated on the surface membrane of these cells and not in the lumen as hitherto thought. However, considerable enterokinase activity can be demonstrated in human duodenal juice after pancreozymin stimulation, indicating that the anatomical and functional localization of enterokinase are perhaps not identical (Hadorn et al., 1971). The activity of enterokinase in the lumen is proportionally larger (compared with the activity of the mucosa) than the activity of other brush-border enzymes such as sucrase and maltase, both in the fasting state and after a test meal (Nordström & Dahlqvist, 1971a and unpublished work in collaboration with A. Dahlqvist and Å. Nordén). Also some other facts made it doubtful that the functional localization of enterokinase was on the mucosal surface. Sucrase, maltase, leucyl naphthylamidase, and other previously known brush-border enzymes are involved in terminal digestion of foodstuffs, and their substrates are small molecules. Several of the enzymes have also been clearly demonstrated to work in close structural and functional relation to subsequent absorption processes within the membrane (Crane, 1969). In contrast, the activatory proteolysis of trypsinogen represents an initial step in the sequence of digestive events. Trypsinogen is a high molecular-weight substrate, and the nature of the substrate is not a foodstuff but a precursor of a digestive enzyme from an organ other than the intestine. Owing to the large molecule, trypsinogen could be expected to have great difficulties in reaching the enterokinase at the membrane. It was therefore clearly possible that enterokinase was released by some specific mechanism and that trypsinogen was activated in the lumen. The association of the enterokinase activity with the brush border would then reflect an intermediate step in the translocation of the enzyme from the cell interior to the lumen.

In paper VI and VII, investigations were undertaken to clarify the release mechanism and magnitude of the luminal enterokinase activity. It was hypothesized that either some luminal factor (pancreatic proteases or bile) or hormonal factor could be involved. Also experiments aimed at estimating and comparing the capacity for activation of trypsinogen by the luminal enterokinase activity and by the enzyme bound to the mucosal surface,

respectively, were carried out (to be published). The results of these experiments will be discussed below in this context.

Release from isolated duodenal brush borders

In paper VI, the release of enterokinase from isolated rat duodenal brush borders by various enzymes (especially pancreatic proteases) and bile salts was studied and compared with the release of other enzyme activities present in the brush border (lactase, sucrase, isomaltase, maltase, trehalase, alkaline phosphatase and leucyl naphthylamidase). Papain released the enterokinase activity more easily than any of the other enzyme activities. Trypsin and chymotrypsin both caused a significant release of enterokinase activity but no or negligible release of the other enzyme activities. But when these proteases were incubated with a homogenate prepared in a way that disintegrates the brush borders into small fragments, they were able to solubilize both enterokinase and a number of disaccharidases. Carboxypeptidase and hyaluronidase did not release any brush-border enzymes. Bile salts released enterokinase activity but also other enzyme activities in equivalent amounts.

These enzyme-dissection experiments allowed some conclusions about the morpho-chemical relation between enterokinase and other brush-border enzymes and between enterokinase and the membrane (see section on Anatomical localization, subcellular localization). They also suggested that trypsin and chymotrypsin might contribute to create the soluble enterokinase activity present in the lumen. Both proteases caused a release in concentrations well below, as well as around, physiological. Their proteolytic action on the surface membrane of intact and/or predesquamated cells of the villi could explain why the duodenal juice contains more enterokinase than disaccharidase activity; it could also account for the soluble form of enterokinase present in the juice. The fact that trypsinogen must be activated by enterokinase (and chymotrypsinogen by trypsin) before the proteases become active was no argument against such a mechanism, because the trypsinogen is partly activated by enterokinase while this enzyme is still associated with the intestinal wall (unpublished observation). Furthermore, enterokinase is liberated from the mucosa in desquamated cells which, after disintegration, will make the enterokinase as accessible as in the isolated brush borders. But it was obvious that experiments using more physiological methods were needed before the importance of pancreatic proteases for the luminal enterokinase activity *in vivo* could be finally evaluated. None the less, the findings were very challenging, as they were consistent with old statements by Pavlov (1907) that enterokinase is secreted from the intestinal wall on direct stimulation by pancreatic proteases. Pavlov's observation had also recently been confirmed by Lepkovsky *et al.* (1970) in studies on the luminal enterokinase activity of chickens in the presence and in the absence of active pancreatic proteases.

Bile salts were also found to solubilize part of the enterokinase activity present in isolated brush border, in agreement with the findings by Hadorn

et al. (1971), who reported that bile salts might be responsible for a physiological release of enterokinase from the intact cells of the villi. The bile-salt effect was found not to be specific, however, as also other brush-border enzyme activities were released to at least the same extent. It could also be questioned whether the bile-salt effect was a true solubilization or rather mainly a formation of low specific weight aggregates with relatively large membrane fragments.

Release from the small-intestinal wall

The release of enterokinase, alkaline phosphatase, trehalase, maltase, and sucrase activities from the small-intestinal wall was systematically studied in vivo in gut segments in rat. First, the release following combined cholecystokinin and secretin stimulation was determined. Then the release effect of different factors (bile, bile salts, pancreatic juice, pancreatic proteases, and different gastrointestinal hormones) was tested in separate experiments (paper VII).

The elaborated in vivo technique included anaesthesia with a cyclopropane-oxygen mixture through a tracheotomy tube. This anaesthesia was more easy to control than that with ether or barbiturate and the tracheotomy also facilitated bronchial suction. Some perfusion experiments were performed, but it was more advantageous to employ a closed segment technique in most experiments. The abdomen was opened by a midline incision, and a thin inlet cannula was inserted through a small incision in the duodenum. It was tied in place with a ligature immediately proximal or distal to the common bile and pancreatic duct opening, depending on whether or not pancreatic and bile secretions into the segment was required during the subsequent experiment. The distal end of the experimental segment (3-4 cm unstretched tissue) was made by tying off the intestine. Luminal factors to be tested were introduced into the lumen in an amount that gave moderate filling of the segment. Hormones were injected through the femoral vein.

Table I shows the enzyme release after strong, combined stimulation with cholecystokinin and secretin in segments with intact bile and pancreatic secretions. The released amount of each activity is expressed as per cent of the total intestinal activity, i.e. the sum of the activity in the lumen and the activity remaining in the wall. About 15 % of the total enterokinase and alkaline phosphatase activities were present in the lumen 35 minutes after hormone stimulation, whereas the released amount of sucrase was only about 7,5 %. Maltase and sucrase had intermediate release figures. Only minor fractions of the enzyme amounts were present in the lumen at zero time. Visual inspection during the experiment revealed that the segment became filled with fluid (yellow from bile) and that the motility increased strongly.

It was further investigated whether the enzyme release following cholecystokinin and secretin stimulation might be caused by an action on the small-intestinal wall of factors present in the bile and pancreatic secretions or whether the hormones act more directly.

TABLE I

Enzyme release after cholecystokinin and secretin stimulation
(bile and pancreatic secretions intact)

A combined dose of cholecystokinin and secretin (4-6 units/kg of each hormone) was given intravenously. The experiment was finished 35 min later. The released amount of each enzymic activity is expressed as percent of the total intestinal activity, i.e. the sum of the activity in the lumen and the activity remaining in the wall (from paper VII).

Enzymic activity	Release (%) in experiment					Mean
	1	2	3	4	5	
Enterokinase	17.1	15.6	11.6	15.8	12.9	14.6
Sucrase	6.6	9.5	6.0	9.7	5.7	7.5
Trehalase	9.6	13.8	7.5	8.7	9.5	9.8
Maltase	9.4	14.7	9.3	12.6	7.7	10.7
Alkaline phosphatase	15.8	21.6	9.4	16.1	14.6	15.5

Introduction of bile into intestinal segments lacking the common bile and pancreatic duct caused a release that resembled the results obtained after hormone stimulation (Table I), both regarding the ratios and the absolute amounts of released enzyme activities. Perfusions with bile salt mixtures in saline (physiological concentrations) and with saline alone showed that the presence of bile salts increased the output of enterokinase and also of other enzyme activities several times.

Also pancreatic juice (containing activated proteases) and pure pancreatic proteases caused a significant release of the enzymes studied, the ratios for the released amounts being similar to that of Table I. A release was also observed in rats with bile fistula after cholecystokinin and secretin stimulation.

Even in the absence of bile and pancreatic secretions, stimulation with cholecystokinin and/or secretin caused a rise in the luminal content of brush-border enzymes. However, the release values were lower than those obtained after stimulation in animals with intact bile and pancreatic secretions. Cholecystokinin was more active than secretin. The ratios for the released amounts of enzyme activities were similar to those of the other experiments. Gastrin and glucagon caused no clear release.

After cholecystokinin and secretin stimulation, the concentrations of trypsin and chymotrypsin found in the lumen were lower in rats with bile fistula than in animals with intact bile secretion. This indicates that the activation of the enterokinase activity caused by the bile salts present in

the secreted bile is of physiological importance at least in the rat.

In all the different types of release experiments *in vivo* enterokinase behaved in the same way as other brush-border enzymes both quantitatively and qualitatively. This was a new strong indication that the presence of this enzyme in the brush borders of the villous epithelial cells is not due to an adsorption secondary to secretion from another site in the intestine. The released amounts of the enzymes were always a minor fraction of the total intestinal activity. The rise in luminal enterokinase activity after cholecystokinin and secretin stimulation in the intact rat (Table I) seems to be partly an effect of a direct action of bile salts and proteases on the intestinal wall. Especially cholecystokinin contributes to the release also in another way, through a direct action on the intact cells of the villi, or indirectly through the strongly increased motility. At least part of the action of bile and proteases (and motility) is on cell fragments trapped in the surface-mucus coating and on cells in a pre-desquamated condition in the tips of the villi. The quantitative differences in the release of different enzymes could well be explained by the differences in their distribution profiles along the villi. The alkaline phosphatase, enterokinase and trehalase activities, which have shown the highest release values, are located more distinctly towards the tips of the duodenal villi than the sucrase and maltase activities.

Stimulation with cholecystokinin and secretin also caused an enzyme release in the absence of bile and pancreatic juice, but it was smaller than the release found with intact bile and pancreatic secretions. In view of all present findings, this release is probably not caused by a direct hormone action on the surface membrane of the intact cells of the villi. It is more probable that it results from the influence of increased motility and fluid secretion on desquamated cell material and on cells in a pre-desquamating condition at the tips of the villi. There seems to exist a population of cells at the top of the starved villus which are ready to be shed in response to feeding (R. Clarke, personal communication).

Recently, it was suggested that bile salts might be responsible for a physiological release of enterokinase from the intact cells of the villi, as judged from experiments with isolated rat brush borders (Hadorn *et al.*, 1971). The present study shows that bile salts are able to release *in vivo*, but this effect is unspecific, as other brush-border enzymes are released in equivalent amounts. Moreover, it is doubtful whether the bile salts in fact do act by releasing enzymes from the intact cells on the villi.

That pancreatic proteases cause a release of enterokinase activity from the intestinal wall is in agreement with previous observations by Pavlov (1907) and Lepkovsky *et al.* (1970). However, the present study has

revealed that other brush-border enzymes are released in equivalent amounts. Moreover, the pancreatic proteases are not the only release factor and they are probably less important than bile salts under physiological conditions. The release by trypsin and chymotrypsin does not seem to be due to an enzyme-solubilizing action on the membrane of the intact cells of the villi, because these proteases cannot solubilize any brush-border enzymes except enterokinase (isolated brush borders).

In parallel work, Götze *et al.* (1972) have studied the release of enterokinase and other brush-border enzymes from the small-intestinal wall in the rat. An increase in the concentrations of brush-border enzymes including enterokinase was observed in the perfusate after cholecystokinin stimulation. No information was given about the released amounts of enzymes in relation to the activities remaining in the wall. The authors attributed the release to a direct hormonal stimulation of the gut wall but did not exclude the possibility of desquamation as an enzyme-releasing mechanism.

Perfusion experiments with trypsinogen in vivo

The *in vivo* technique was the same as described above. A solution containing trypsinogen was perfused through the duodenal segment (situated distal to the common bile and pancreatic duct) at constant flow rate (usually 6.6 ml/hour) and the amount of trypsin in the outflow was determined. The inflow solution contained bovine trypsinogen (0.3 or 0.8 mg/ml) in 0.01 M maleate buffer, pH 6.2, made isotonic with sodium chloride. In addition, sodium taurodeoxycholate (2.6 mM) was added in some experiments. The intestinal segment was washed through with buffer alone before the *in vivo* activity of enterokinase was estimated (trypsin was undetectable in the outflow at the end of the washing). At the end of the experiment, the intestinal segment was homogenized and the enterokinase activity was determined *in vitro* with incubation conditions identical to those present during the *in vivo* perfusion.

Fig. 11 shows that a small fraction of the trypsinogen load was activated (in the order of 5-10 % assuming that the true load is about 0.5 mg/ml, 0.3 mg/ml being made up of MgSO_4). The activation capacity did not change very much during the experimental period (1 hour and 40 min.). About 10 times more trypsin was formed, when the enterokinase activity of the intestinal segment was determined after homogenization. A surprising finding was that perfusions with trypsinogen in the absence of bile salts gave a trypsin concentration in the outflow of the same order as when bile salt was present. The enterokinase activity in the perfusate was too low to be detectable after gel filtration. There was no evidence that the trypsin activity increased to an important extent while in the collection tubes or during the assay of trypsin activity, which was carried out immediately at the end of the experiment.

The *in vivo* perfusions involve many unavoidable factors that can influence on the results. They will not be discussed in detail in this context. But most of these factors will (if active) tend to produce a false high value for

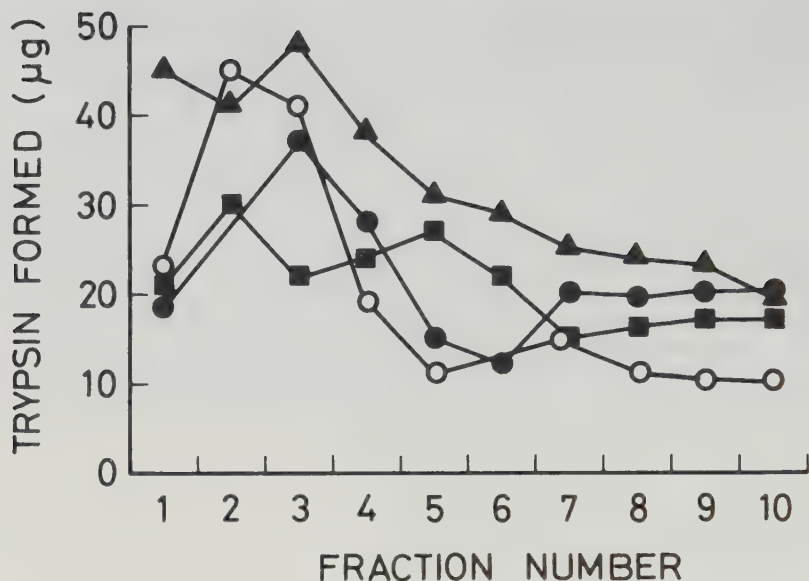


Fig. 11 The amount of trypsin in the outflow, when a duodenal segment was perfused with trypsinogen (0.8 mg/ml) in the presence of sodium deoxycholate (2.6 mM) at a rate of 6.6 ml/hour. Experiments with 4 rats were carried out. Collection time: 10 min per fraction (unpublished work).

the *in vivo* activation of trypsinogen. Therefore the present results make it a reasonable assumption that the released amount of enterokinase activity after cholecystokinin stimulation (about 15 % of the total intestinal activity) can account for at least the same activation as the remaining activity in the wall. The large (and charged) trypsinogen molecules might have difficulties in reaching the enterokinase at the membrane rapidly and easily due to the mucus layer covering the mucosal surface of the intestine. Also glycocalyx can constitute a partial barrier provided that enterokinase is covered by it and not part of it. Furthermore, all membrane-bound enterokinase may not be accessible to the trypsinogen. Only part of the enterokinase activity of isolated brush borders was released by trypsin and chymotrypsin (VI).

The present study seems to exclude the existence of a dramatic and conventional secretion of enterokinase as a result of a direct or indirect action of gastrointestinal hormones. Enterokinase seemed to mainly reach the lumen together with other brush-border enzymes in large fragments

or in whole desquamated cells, the desquamation being promoted by several factors. None the less, there is some evidence that enterokinase more easily leaves its site at the membrane than do other enzymes in vivo, as found in vitro (VI):

1. Perfusions with saline in rats invariably yielded more enterokinase than any other brush-border enzyme in the outflow (VII and unpublished work). However, the absolute amounts of activity were small.
2. Perfusions with trypsin or chymotrypsin in saline tended to increase this discrepancy.
3. In the intestinal lumen of the fasting rat, there is always more enterokinase than other brush-border enzymes (VII). This discrepancy is much more expressed in man. The specific enterokinase activity present in duodenal juice from fasting patients is in the same order as the corresponding activity in proximal duodenal biopsy specimens from the same patients (unpublished observations by the author in collaboration with Å. Nordén). In contrast, the specific alkaline phosphatase activity of the juice is much lower (a few per cent to 20 per cent) than the specific activity in biopsy specimens. The maltase and sucrase activities in the juice are almost negligible compared to the activities in the wall. It is quite possible that the considerable luminal enterokinase activity in the fasting man has a physiological significance.

These findings would be consistent with the hypothesis that there is a continuous, slight release of enterokinase from the membrane and into the lumen in steady state with a synthesis within the cell. Speculatively, enterokinase, a glycoprotein, might be completed in the Golgi apparatus, transported to the microvillous membrane, incorporated in the membrane on its external side (or in the glycocalyx) and turned over, this turnover involving the release of the enzyme into the lumen. Such a concept is stimulating as a working hypothesis in view of recent progress in membrane research. Migration of glycoproteins from Golgi apparatus to the cell surface takes place in the intestinal epithelium (Ito, 1969; Benett, 1970). Glycoproteins are only present on the external side of the cell membrane (Steck, 1972). Components of the brush-border membrane, including enzymes such as the disaccharidases, are turned over within the life time of the cell, a more rapid turnover being found for large and superficially located proteins (Alpers, 1971). Glycoprotein secretion has been reported to take place from intestinal slices (Lukie & Forstner, 1972).

The proposed hypothesis could also explain the finding that the enterokinase activity is not as reduced as the disaccharidases in patients with coeliac disease. If the membrane of the cells is destroyed, possible the enterokinase accumulates in the cells leading to much higher concentration of enterokinase per cell than in normals. The extreme resistance to proteolysis exhibited by enterokinase (Yamashina, 1956a) would help the enzyme to escape degradation intracellularly (cf. Schimke & Dehlinger, 1972) as it probably prevents degradation extracellularly in the duodenal juice.

SUMMARY

A method was developed for the isolation of different parts of the villi and crypts, which permitted quantitative analyses of the enzyme activities of narrow cell layers from the tip of the villi to the bottom of the crypts. Various small-intestinal enzymes, especially the digestive, were studied in this way in rat and human intestine. Digestive enzymes were almost exclusively present along the villi with highest activities in the mid-villi or apical halves of the villi. Within the villi, interesting differences in their distribution patterns were seen. Lysosomal enzymes had a rather flat distribution profile with about the same activities along the villi and crypts. Thymidine kinase, an enzyme related to cellular proliferation, was mainly present in the crypts. The obtained distribution patterns show how different enzymic properties appear and change quantitatively with age and stage of differentiation of the epithelial cells. Such knowledge gives a new aspect in our concept of the functional organization of the mucosa. The findings are discussed with regard to present knowledge of the enzymes in the normal and diseased gut.

One intestinal enzyme, the enterokinase (enteropeptidase, EC 3.4.4.8), the activator of the trypsinogen from pancreas, was subjected to a more extensive study, because it had been little investigated in spite of its vital importance for the utilization of dietary protein. The localization and the properties of the enzyme were studied in rat and man. The mechanism of the release of enterokinase from the intestinal wall and the site of its physiological function were given especial attention.

Highest activity of rat enterokinase was found in duodenum, the activity decreasing sharply in distal direction. Human enterokinase activity was present in all parts of duodenum. Within the intestinal wall, activity was present in the villi but essentially absent from the crypts. High activity was present in the cells in the tips of the villi. No activity was found in the Brunner glands. The subcellular localization of enterokinase was in the superficial brush-border region of the villous epithelial cells.

These results are surprising in view of the general concept of enterokinase as a secretion product from deep layers of the mucosa. They imply that enterokinase is formed in the absorptive cells of the villus and incorporated in their surface membrane. This means a principally new functional property of the brush-border organelle, because enterokinase differs so fundamentally from previously known brush-border enzymes both concerning the type of substrate and the point of action in the sequence of digestive events.

The enterokinase activity was activated by bile salts, whereas increasing the ionic strength greatly depressed the activity. No important metal ion activators were found.

To obtain information on the morpho-chemical relation between enterokinase and well-known brush-border enzymes and between enterokinase and the membrane, isolated brush borders were treated with proteolytical enzymes. Trypsin and chymotrypsin caused a selective release of enterokinase, and papain released enterokinase more easily than it released any other enzyme from the membrane. The results are in accord with the concept that enterokinase is superficially located in the membrane and that the brush-border enzymes are organized within the membrane in a mosaic-like pattern.

The release of enterokinase and other brush-border enzymes from the small-intestinal wall was studied in vivo in gut segments in rat to clarify the release mechanism and magnitude of the luminal enterokinase activity. Both the effect of luminal (bile, pancreatic proteases) and hormonal factors was investigated. In all experiments enterokinase behaved in the same way as other brush-border enzymes. The rise in luminal enterokinase activity after cholecystokinin and secretin stimulation in the intact rat (15 % of the total intestinal activity) seemed to be partly an effect of a direct action of bile salts and proteases on the intestinal wall. Cholecystokinin contributed to the release also in another way, through the strongly increased motility or possibly more directly. At least part of the action of bile salts and proteases (and motility) is on cell fragments and on cells in a pre-desquamated condition in the tips of the villi.

When an intestinal segment was perfused in vivo with a trypsinogen solution, trypsin was produced. But the amount of trypsin formed was about 10 times greater, when the enterokinase activity of the segment was determined after homogenization. Consequently, after hormone stimulation the luminal enterokinase activity probably can bring about a better activation of the trypsinogen than the total remaining activity in the wall.

The physiological cell desquamation is one mechanism by which enterokinase reaches the lumen. No conventional secretory mechanism seems to exist. But there is some evidence that enterokinase more easily leaves its site at the membrane than do other enzymes in vivo, as found in vitro. There might be a continuous, slight release of this enzyme from the membrane and into the lumen in steady state with a synthesis within the cell.

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REFERENCES

- ALPERS, D.H. (1972) The relation of size to the relative rates of degradation of intestinal brush border proteins. J. Clin. Invest. 51: 2621-2630.
- ASP, N.-G. (1971) Small-intestinal β -galactosidases. Characterization of different enzymes and application to human lactase deficiency. Studentlitteratur, Lund.
- ASP, N.-G., KOLDOVSKÝ, O. & HOŠKOVÁ, J. (1968) β -Galactosidase activity of the jejunum and ileum of newborn, suckling and weaned rats. Physiol. bohemoslov. 17: 229-236.
- BAINTNER, JR. K. & VERESS, B. (1970) Complex changes in the suckling rat's small intestine by the end of the third week of life. Experientia 26: 54-55.
- BAYLISS, W.M. & STARLING, E.H. (1904) The proteolytic activities of the pancreatic juice. J. Physiol. 30: 61-83.
- BENNETT, G. (1970) Migration of glycoprotein from Golgi apparatus to cell coat in the columnar cells of the duodenal epithelium. J. Cell Biol. 45: 668-673.
- DE BOTH, N.J., VAN DER MEER, W. & GALJAARD, H. (1972) Microchemical studies on cell regeneration in the small intestine. 9^e Congrès international de gastroentérologie. Archives Francaises des Maladies de l'Appareil Digestif 61: N^o 6-7, p. 311c.
- BROWN, A.L. (1962) Microvilli of the human jejunal epithelial cell. J. Cell Biol. 12: 623-627.
- CLARKE, R.M. & HARDY, R.N. (1969) An analysis of the mechanism of cessation of uptake of macromolecular substances by the intestine of the young rat ("closure"). J. Physiol. 204: 127-134.
- CORNELL, R. & PADYKULA, H. (1969) A cytological study of intestinal absorption in the suckling rat. Am. J. Anat. 125: 291-316.
- CRANE, R.K. (1966) Structural and functional organization of an epithelial cell brush border. In Intracellular transport, edited by K.B. Warren, Vol. 5, p. 71-103. Academic Press, New York.
- CRANE, R.K. (1968) Digestion and absorption of carbohydrates. In Carbohydrate metabolism and its disorders, edited by F. Dickens, P.J. Randle and W.J. Whelan, Vol. 1, p. 25-51. Academic Press, London and New York.
- CRANE, R.K. (1969) A perspective of digestive-absorptive function. Am. J. Clin. Nutr. 22: 242-249.
- DAHLQVIST, A. (1964) Intestinal disaccharidases, p. 7-55. In Disorders due to intestinal defective carbohydrate digestion and absorption, edited by P. Durand. Il Pensiero Scientifico, Rome.

- DAHLQVIST, A. & NORDSTRÖM, C. (1966) The distribution of disaccharidase activities in the villi and crypts of the small-intestinal mucosa. Biochim. Biophys. Acta 113: 624-626.
- DAS, B.C. & GRAY, G.M. (1969) Protein synthesis in small intestine: localization and correlation with DNA synthesis and sucrase activity. Biochim. Biophys. Acta 195: 255-257.
- DASTRE, A. & STASSANO, H. (1904) Les facteurs de la digestion pancréatique. Suc pancréatique, kinase et trypsine, anti-kinase Arch. Internat. Phys. 1: 86-117.
- DAVIE, E.W. & NEURATH, H. (1955) Identification of a peptide released during autocatalytic activation of trypsinogen. J. Biol. Chem. 212: 515-529.
- DAWSON, I. & PRYSE-DAVIES, J. (1963) The distribution of certain enzyme systems in the normal human gastrointestinal tract. Gastroenterology 44: 745-760.
- DELEZENNE, M.C. (1902a) Sur la distribution et l'origine de l'entérokinase. C.R. Soc. Biol. 54: 281-283.
- DELEZENNE, M.C. (1902b) Sur la présence dans les leucocytes et les ganglions lymphatiques d'une diastase favorisant la digestion tryptique des matières albuminoïdes. C.R. Soc. Biol. 54: 283-285.
- EGGERMONT, E., MOLLA, A.M. & RUTGEERTS, L. (1971) The source of human enterokinase. Lancet II: 369.
- EICHHOLZ, A. (1968) Studies on the organization of the brush border in intestinal epithelial cells. V. Subfractionation of enzymatic activities of the microvillus membrane. Biochim. Biophys. Acta 163: 101-107.
- EICHHOLZ, A. (1969) Fractions of the brush border. Gastroenterology 28: 30-34.
- EICHHOLZ, A. & CRANE, R.K. (1965) Studies on the organization of the brush border in intestinal epithelial cells. I. Tris disruption of isolated hamster brush borders and density gradient separation of fractions. J. Cell Biol. 26: 687-691.
- ERLANGER, B., KOKOWSKY, N. & COHEN, W. (1961) The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95: 271-278.
- FALLOISE, A. (1904-05) Distribution et origine des ferments digestifs de l'intestin grêle. Arch. Internat. Phys. 2: 299-321.
- FLOREY, H.W., WRIGHT, R.D. & JENNINGS, M.A. (1941) The secretions of the intestine. Physiol. Rev. 21: 36-69.
- FORTIN-MAGANA, R., HURWITZ, R., HERBST, J.J. & KRETCHMER, N. (1970) Intestinal enzymes: Indicators of proliferation and differentiation in the jejunum. Science 167: 1627-1628.
- FRÍČ, P., LOJDA, Z., JODL, J. & MALÍŠ, F. (1969) Analysis of peroral jejunal biopsies in clinically asymptomatic parents of children with coeliac sprue. Digestion 2: 35-42.

- GALJAARD, H., BUYS, J., VAN DUUREN, M. & GIESEN, J. (1970) A quantitative histochemical study of intestinal mucosa after X-irradiation. J. Histochem. Cytochem. 18: 291-301.
- VAN GENDEREN, H. & ENGEL, C. (1938) On the distribution of some enzymes in the duodenum and ileum of the rat. Enzymologica 5: 71-80.
- GOLDMAN, R.B., KIM, Y.S., JONES, R.S. & SLEISENGER, M.H. (1971) The effect of glucagon on enterokinase secretion from Brunner's gland pouches in dogs. Proc. Soc. Exp. Biol. Med. 138: 562-565.
- GÖTZE, H., ADELSON, J.W., HADORN, H.B., PORTMANN, R. & TROESCH, V. (1972) Hormone-elicited enzyme release by the small intestinal wall. Gut 13: 471-476.
- GREY, R.D. & LECOUNT, T. (1970) Distribution of leucyl naphthylamidase and alkaline phosphatase on the villi of the chick duodenum. J. Histochem. Cytochem. 18: 416-423.
- HADORN, B. (1969) Studies on human enterokinase. Ph. D. Thesis, University of Melbourne.
- HADORN, B., STEINER, N., SUMIDA, C. & PETERS, T.J. (1971) Intestinal enterokinase: Mechanisms of its "secretion" into the lumen of the small intestine. Lancet I: 165-166.
- HADORN, B., SUMIDA, C. & TROESCH, V. (1972) Biochemical investigations in four patients with intestinal enterokinase deficiency. 9^e Congrès international de gastro-enterologie. Archives Françaises des Maladies de l'Appareil Digestif 61: N° 6-7, p. 156c.
- HADORN, B., TARLOW, M.J., LLOYD, J.K. & WOLFF, O.H. (1969) Intestinal enterokinase deficiency. Lancet I: 812-813.
- HAMBURGER, H.J. & HEKMA, E. (1902) Sur le suc intestinal de l'homme. Journ. de Physiol. et Pathol. gén. 4: 805-819.
- HEIDENHAIN, R. (1875) Beiträge zur Kenntniss des Pankreas. Pflüger Arch. Ges. Physiol. 10: 557-632.
- HERBST, J.J., FORTIN-MAGANA, R. & SUNSHINE, P. (1970) Relationship of pyrimidine biosynthetic enzymes to cellular proliferation in rat intestine during development. Gastroenterology 59: 240-246.
- HERBST, J.J. & KOLDOVSKÝ, O. (1972) Cell migration and cortisone induction of sucrase activity in jejunum and ileum. Biochem. J. 126: 471-476.
- HERINGOVÁ, A., JIRSOVÁ, V. & KOLDOVSKÝ, O. (1965) Postnatal development of β -glucuronidase in the jejunum and ileum of rats. Canad. J. Biochem. 43: 173.
- HOLMES, R. & LOBLEY, R.W. (1970) The localization of enterokinase to the brush-border membrane of the guinea pig small intestine. Proc. Physiol. Soc. J. Physiol. 211: 50P-51P (abstract).
- HOLTER, H. & LINDERSTRÖM-LANG, K. (1934) Beiträge zur enzymatischen Histochemie. IX. Die Verteilung des Pepsins in der Schleimhaut des Schweinemagens. Hoppe-Seylers Z. Physiol. Chem. 226: 149-172.

- IMONDI, A.R., BALIS, M.E. & LIPKIN, M. (1969) Changes in enzyme levels accompanying differentiation of intestinal epithelial cells. Exptl Cell Res. 58: 323-330.
- ITO, S. (1969) Structure and function of the glycocalyx. Gastroenterology 28: 12-25.
- JAMES, W.P.T., ALPERS, D.H., GERBER, J.E. & ISSELBACHER, K.J. (1971) The turnover of disaccharidases and brush border proteins in rat intestine. Biochim. Biophys. Acta. 230: 194-203.
- KOLDOVSKÝ, O. & CHYTIL, F. (1965) Postnatal development of β -galactosidase activity in the small intestine of the rat. Effect of adrenalectomy and diet. Biochem. J. 94: 266-270.
- KOLDOVSKÝ, O., HERINGOVÁ, A. & JIRSOVÁ, V. (1966) β -Galactosidase activity of the jejunum and ileum of suckling rats. Comparison of activities of β -galactosidase at different concentration of substrates (o-nitrophenyl- β -D-galactoside and lactose) at different pH. Biol. neonat. 10: 241-253.
- KÜHNE, W. (1867) Über die Verdauung der Eiweissstoffe durch den Pankreassaft. Virchows Arch. Pathol. Anat. Physiol. 39: 130-173.
- KUNITZ, M. (1939a) Formation of trypsin from crystalline trypsinogen by means of enterokinase. J. Gen. Physiol. 22: 429-446.
- KUNITZ, M. (1939b) Purification and concentration of enterokinase. J. Gen. Physiol. 22: 447-450.
- KUNITZ, M. & NORTHROP, J.H. (1935) Crystalline chymo-trypsin and chymo-trypsinogen. I. Isolation, crystallization, and general properties of a new proteolytic enzyme and its precursor. J. Gen. Physiol. 18: 433-458.
- KUNITZ, M. & NORTHROP, J.H. (1936) Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor, and an inhibitor-trypsin compound. J. Gen. Physiol. 19: 991-1007.
- LEBLOND, C.P. & STEVENS, C.E. (1948) The constant renewal of the intestinal epithelium in the albino rat. Anat. Rec. 100: 357-371.
- LEPKOVSKY, S., FURUTA, F., DIMICK, M.K. & YAMASHINA, I. (1970) Enterokinase and the chicken pancreas. Poultry Science 49: 421-426.
- LOJDA, Z. & MALÍŠ, F. (1972) Histochemical demonstration of enterokinase. Histochemie 32: 23-29.
- LUKIE, B.E. & FORSTNER, G.G. (1972) Synthesis of intestinal glycoprotein incorporation of $[1 - ^{14}\text{C}]$ glucosamine in vitro. Biochim. Biophys. Acta 261: 353-364.
- MAROUX, S., BARATTI, J. & DESNUELLE, P. (1971) Purification and specificity of porcine enterokinase. J. Biol. Chem. 246: 5031-5039.
- MELLANBY, J. & WOOLLEY, V.J. (1913-14) The ferments of the pancreas. Part III. The properties of trypsin, trypsinogen and enterokinase. J. Physiol. 47: 339-360.

- MILLER, D. & CRANE, R.K. (1961) A procedure for the isolation of the epithelial brush border membrane of hamster small intestine. Anat. Biochem. 2: 284-286.
- MILSTONE, J.H., GOLDBLAT, M.V. & MILSTONE, V.K. (1969) Physiologic fitness of enterokinase. Proc. Soc. Exptl Biol. Med. 131: 1438-1441.
- MOOG, F. & GREY, R.D. (1967) Spatial and temporal differentiation of alkaline phosphatase on the intestinal villi of the mouse. J. Cell Biol. 32: C1-C6.
- MOOG, F. & GREY, R.D. (1968) Alkaline phosphatase isoenzymes in the duodenum of the mouse: Attainment of pattern of spatial distribution in normal development and under the influence of cortisone or actinomycin D. Develop. Biol. 18: 481-500.
- NASSET, E.S. & JU, J.S. (1970) Intestinal enzyme secretion in guinea pig as affected by extract of gut mucosa. Fed. Proc. 29: 722 Abs.
- NORDSTRÖM, C. & DAHLQVIST, A. (1970) The cellular localization of enterokinase. Biochim. Biophys. Acta 198: 621-622.
- NORDSTRÖM, C. & DAHLQVIST, A. (1971a) The cellular and sub-cellular localization of enterokinase. Acta Paediat. Scand. 60: 369-370.
- NORDSTRÖM, C. & DAHLQVIST, A. (1971b) Intestinal enterokinase. Lancet I: 1185-1186.
- NORDSTRÖM, C. & DAHLQVIST, A. (1973) Enterokinase: Localization and physiological function. Symposium on Intestinal enzyme deficiencies, Swedish Nutrition Foundation, Saltsjöbaden, August 1972, Almquist & Wiksell, Stockholm. In press.
- PAVLOV, I.P. (1907) In Les Prix Nobel en 1904. P.A. Norstedt & Söner for the Nobel Foundation, Stockholm.
- PAVLOV, I.P. (1910) The work of the digestive glands. Charles Griffin, London.
- POTTER, V.R. & ELVEHJEM, C.A. (1936) A modified method for the study of tissue oxidations. J. biol. Chem. 114: 495-504.
- REY, J., SCHMITZ, J., REY, F. & JOS, J. (1971) Cellular differentiation and enzymatic deficits. Lancet II: 218.
- ROVERY, M., FABRE, C. & DESNUELLE, P. (1952) Étude des extrémités N-terminales du trypsinogène et de la trypsine de boeuf. Biochim. Biophys. Acta 9: 702.
- RUTGEERTS, L., TYTGAT, G. & EGGERMONT, E. (1972) Localization of enterokinase in man. Gastroenterology 63: 381.
- SCHEPOWALNIKOV, N.P. (1899) Physiology of the intestinal juice. (Russian.) Thesis, St. Petersburg. Summary in Maly's Jahresbericht über die Fortschritte der Thier-Chemie 29 (1900): 378-380.
- SCHIMKE, R.T. & DEHLINGER, P.J. (1972) Turnover of membrane proteins of animal cells, pp. 115-133. In Membrane Research, edited by C.F. Fox. Academic Press, New York and London.

- SINGER, S.J. & NICOLSON, G.L. (1972) The fluid mosaic model of the structure of cell membranes. Science 175: 720-727.
- STECK, T. (1972) The organization of proteins in human erythrocyte membranes, pp. 77-93. In Membrane Research, edited by C.F. Fox. Academic Press, New York and London.
- SYLVÉN, C. & BORGSTRÖM, B. (1969) Absorption and lymphatic transport of cholesterol and sitosterol in the rat. J. Lipid Res. 10: 179-182.
- SYLVÉN, C. & NORDSTRÖM, C. (1970) The site of absorption of cholesterol and sitosterol in the rat small intestine. Scand. J. Gastroent. 5: 57-63.
- TAKANO, K., SUZUKI, T. & YASUDA, K. (1971) Immunohistochemical localization of enterokinase in the porcine intestine. Okajimas Fol. anat. jap. 48: 15-28.
- TARLOW, M.J., HADORN, B., ARTHURTON, M.W. & LLOYD, J.K. (1970) Intestinal enterokinase deficiency. A newly-recognized disorder of protein digestion. Arch. Disease Childhood 45: 651-655.
- TRIER, J.S. (1968) Morphology of the epithelium of the small intestine. In Handbook of Physiology, edited by C.F. Code and W. Heidel, Sect. 6, Vol. III, pp. 1125-1175. Baltimore.
- UGOLEV, A.M., IEZUITOVA, N.N., KUSHAK, R.I. & SHCHERBAKOV, G.G. (1970) Invertase and dipeptidase activity distribution in small intestine crypt-villus system. Die Nahrung 14: 437-452.
- VERNON, H.M. (1913-14) The auto-catalysis of trypsinogen. J. Physiol. 47: 325-338.
- WALDSCHMIDT-LEITZ, E. (1923-24) Über Enterokinase und die tryptische Wirkung der Pankreasdrüse. Zs. Physiol. Chem. 132: 181-237.
- WEBSTER, H.L. & HARRISON, D.D. (1969) Enzymic activities during the transformation of crypt to columnar intestinal cells. Exptl Cell Res. 56: 245-253.
- WELSH, J.D., PREISER, H., WOODLEY, J.F. & CRANE, R.K. (1972) An enriched microvillus membrane preparation from frozen specimens of human small intestine. Gastroenterology 62: 572-582.
- WOODLEY, J.F. & KEANE, R. (1972) Enterokinase in normal intestinal biopsies and those from patients with untreated coeliac disease. Gut 13: 900-902.
- YAMASHINA, I. (1956a) Studies on enterokinase. II. The further purification. Arkiv Kemi 9: 225-229.
- YAMASHINA, I. (1956b) The action of enterokinase on trypsinogen. Acta Chem. Scand. 10: 739-743.

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